

STUDIES ON THE RESPONSE OF PLEURAL LEUKOCYTES
TO ASBESTOS EXPOSURE

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**I declare that this thesis was written by me
and that the work contained herein is my own**

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ABSTRACT

Asbestos is a naturally occurring fibrous silicate mineral which has extensive industrial and domestic application. Exposure to asbestos is associated with pulmonary fibrosis and lung cancer. In addition, the pleura is also a major target for the harmful effects of asbestos exposure which include pleural plaques, pleural fibrosis and pleural mesothelioma. These pleural pathological changes severely affect the normal pleural functions. However, in spite of its importance, few studies have addressed the process of fibre-related pleural pathology. The aim of the present study was to assess the pleural response, in particular, the pleural leukocyte response to asbestos fibres following their deposition in the bronchoalveolar space using a rat intratracheal instillation model.

In an attempt to investigate whether asbestos or other particles deposited in the lung could reach the pleural space, a number of approaches were taken: 1). intratracheal instillation of fluorescent beads, which could be easily visualised, alone or in combination with heat-killed *C.parvum* organism causing inflammation; 2). intratracheally instilled asbestos and 3). inhaled asbestos. A combination of light, fluorescent and transmission electron microscopes were then used to assess the transfer of fibres or particles to the pleural space. No evidence could be found for the transfer of any particles or fibres to the pleural space under any conditions.

Normal pleural leukocytes were found to be comprised of macrophages, mast cells, eosinophils and lymphocytes and this population was confirmed to produce urokinase-type plasminogen activator inhibitor (PAI) *in vitro* and to contain plasminogen activator (PA) intracellularly. Normal pleural leukocytes also released tumour necrosis factor (TNF) and interleukin-1 (IL-1) in culture and the release was enhanced further with LPS stimulation.

After intratracheal instillation (I/T) of crocidolite asbestos, pleural leukocyte components were changed as from 3 days after instillation, by a significant recruitment of macrophages and eosinophils. These populations were found to release increased PAI activity and decreased TNF activity in a dose-related manner to the quantity of asbestos instilled; no time-dependent effect was observed. In contrast, I/T asbestos coupled with mineral dusts, titanium dioxide (TiO₂) or quartz, considerably increased TNF production by pleural leukocytes. The elaboration of IL-1 by the leukocytes showed an inverse relationship to asbestos up to 14 days, but was dramatically increased by one month after instillation compared to control.

During the early stage of pleural inflammation caused by *C.parvum* organisms, the pleural leukocytes which contain a substantial neutrophil infiltrate

produced increased PAI activity, but decreased TNF and IL-1 release. By 5 days after I/T *C.parvum*, the pleural leukocytes with a predominant newly- recruited macrophage population, released even less TNF and IL-1, whereas PAI production by the cells recovered to control level.

Studies on the normal pleural lavage fluid indicated that components of the fluid inhibited both TNF and IL-1 activity, which imply a suppressive environment in the pleural space. However, pleural lavage from rats one month after I/T asbestos was shown to stimulate the proliferation of mesothelial cells *in vitro*.

In comparison with pleural leukocytes, after I/T asbestos, bronchoalveolar leukocytes produced increased TNF and IL-1 activity *in vitro* in an asbestos dose-related manner. However, IL-1 production by the alveolar leukocytes showed a transient reduction over control level.

The investigation on interaction of pleural leukocytes and pleural mesothelial cells showed that the pleural leukocytes one day after I/T asbestos caused mesothelial cell detachment injury *in vitro*, whereas the cells from rats longer term after instillation showed no such effect. The leukocytes from *C.parvum*- induced inflamed pleura also caused mesothelial cell detachment injury during the acute phase of inflammation.

In *in vitro* studies, long fibre amosite was demonstrated to have more ability to stimulate TNF and IL-1 production by alveolar macrophages than short fibre amosite or TiO_2 . Immunoglobulin G dramatically enhanced the ability of dusts to stimulate TNF production by the macrophages.

To sum up we have demonstrated, that I/T asbestos causes the following pleural leukocyte changes: 1). long standing recruitment of macrophages; 2). increased PAI secretion; 3). decreased production of TNF but enhanced release of TNF when the fibre and particles are in combination; 4). first decreased then increased IL-1 elaboration; 5). causing transient mesothelial cell detachment *in vitro*. All of these changes occur in the apparent absence of transfer of fibres to the pleural space. These observations may help to elucidate the pathogenesis of some pleural disorders caused by exposure to fibrous dusts.

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INTRODUCTION

1. Asbestos

1.1. Asbestos history and application

The term *asbestos* refers to a family of naturally occurring, flexible, fibrous, hydrous silicate crystalline minerals with a high length-to-thickness ratio (Craighead *et al*, 1982; Michaels and Chissick, 1979).

Asbestos has been known and used in small amounts for thousands of years, but it was not widely used prior to the latter part of the 19th Century (Wright, 1969). World-wide consumption of asbestos during the past 80 years has increased by at least 1000-fold (Buchanan, 1979).

Because of its heat- and fire-resistant properties, as well as its high tensile strength and flexibility, asbestos finds its greatest use in industry. Some 3000 uses for asbestos have been recorded (Rosato, 1959), mainly in the construction industry (about two thirds of the total) (Michaels and Chissick, 1979). These include flat sheets or sidings and corrugated sheets for roofing, rain-water pipes and pressure pipes (Hodgson, 1979).

Second only to its use in building materials, asbestos has a large range of applications, in conjunction with organic resins and similar matrices, such as brake linings and clutch facings. Packings, gaskets, asbestos papers and millboards, spray insulation and electrically resistant reinforced plastics are further examples of the varied applications of asbestos (Hodgson, 1979).

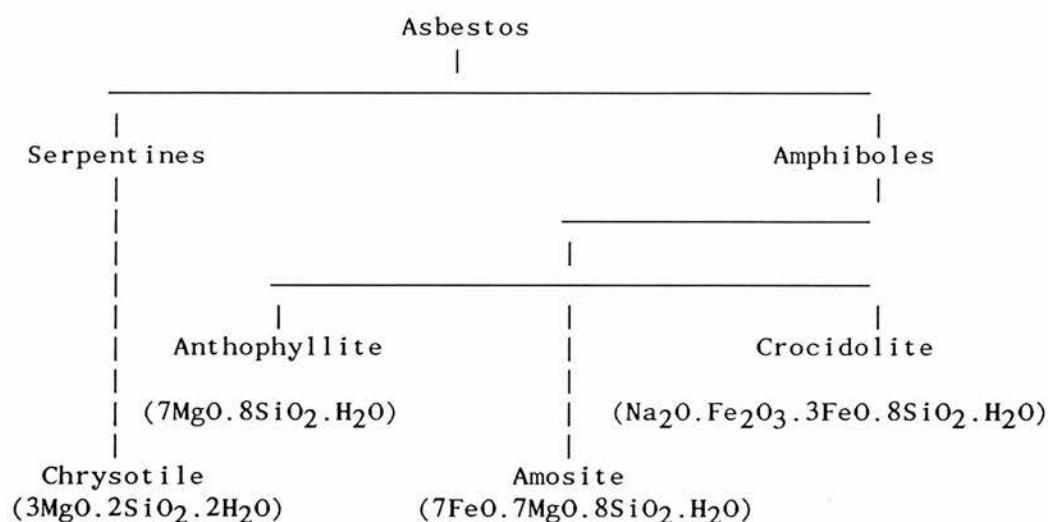
1.2. Asbestos chemistry

Six species of asbestiform minerals are known, these being derived from two large groups of rock-forming minerals, the serpentines and the amphiboles. Chrysotile is the sole species classified in the serpentine group, but it is by far the most abundant kind of asbestos. The amphibole group includes the following asbestiform types: crocidolite, amosite, anthophyllite, tremolite, and actinolite. The mineralogical classification and chemical composition of common commercial asbestos is shown in Figure I.1 (Michaels and Chissick, 1979).

These four main types of asbestos are chemically different: 1. chrysotile or white asbestos, which occurs as fine silky flexible white fibres and is mined mainly in Canada, Russia and Rhodesia; 2. amosite, a straight brittle fibre, light grey to pale brown in colour and found in South Africa; 3. crocidolite or blue asbestos, a straight blue fibre which is found in South Africa, Western Australia, and Bolivia; and 4. anthophyllite, a brittle white fibre mined in Finland and Africa. Other types of asbestos included in the amphibole group are tremolite $[\text{Ca}_2\text{Mg}_5\text{Si}_8\text{O}_{22} \cdot (\text{OH})_2]$ and actinolite $[\text{CaO} \cdot 0.3(\text{MgFe})\text{O} \cdot 4\text{SiO}_2]$ (Michaels and Chissick, 1979; Zussman, 1979).

Chemically, the commercial asbestos minerals are all hydrous silicates. The chemical constitution of the asbestos minerals and their apparent complexities are crystal structures. Chrysotile has a predominant surface of hydroxyl sites connected to an adjacent inner layer of magnesium sites (Hodgson, 1979). In serpentine minerals, the magnesium hydroxide layer has slightly larger dimensions than the silicate (linked SiO_4) layer, resulting in a mis-match, which is resolved in chrysotile into a curvilinear structure of a scroll or coil form. The basic subunit of the amphiboles is a silicon dioxide tetrahedron arranged in parallel chains and linked laterally by various cations (Hodgson, 1979). Amphiboles differ only in the chemical composition of the cation layer sandwiched between the two silicate ribbons.

Figure I.1.



In the electron microscope, chrysotile asbestos has a characteristic appearance, usually as long, thin, curved fibrils. The fine structure of crystalline amphiboles is repeated on a superfine scale in fibrous amphiboles and the cross-section of a fibre bundle has been shown structures skin to the basal cleavage lines in the crystal form (Zussman, 1979; Craighead and Mossman, 1982).

1.3. Asbestos properties

The properties of asbestos include chemical resistance, particularly to acids, fire resistance, tensile strength, and good friction and wear characteristics. Asbestos readily lends itself to a variety of manufacturing processes on account of its wet strength, ease of formation of slurries with water, and good drying characteristics (Michaels and Chissick, 1979; Zussman, 1979).

In view of elemental composition, chrysotile and the amphiboles have

totally different surface properties. Presumably because of the high surface concentrations of magnesium hydroxide, chrysotile fibres exhibit a strong positive surface charge. Amphiboles, by contrast, have a weakly acidic nature and possess a slightly negative surface charge because of cations linked laterally on the fibre surface (Hodgson *et al*, 1979; Craighead and Mossman, 1982).

The various properties of chrysotile and amphibole fibres determine their uses in industry, and in certain respects, may be responsible for their apparently different behaviour in the lungs and in their pathogenic potential (Parkes, 1973). Due to its high flexibility, chrysotile asbestos lends itself mostly to incorporation into textiles (Parkes, 1973). Crocidolite is the strongest of the asbestos fibres and its high mechanical strength combined with its acid resistance makes it a valuable industrial material. The characteristic features of amosite are resistance to corrosion, the springiness of the fibres and so it is widely used for heat insulation applications. Anthophyllite finds use as an expansive filler and in specialised applications on account of its good heat and chemical resistance (Michaels and Chissick, 1979).

Asbestos does not cause a hazard if the fibres are firmly bonded together and incorporated into structures such as asbestos-cement sheets, or into various composite materials. However, because the material is relatively uncontained during the manipulation of asbestos products, high dust levels can occur in processes (Craighead and Mossman, 1982), such as sawing, drilling, mining, processing, manufacturing, installing, transporting, removing, and dumping of any asbestos products (Jones, 1979). Contamination of the environment with asbestos fibres often is subtle but difficult to assess (Harries, 1976; Skidmore and Jones, 1975).

The major pathological effects result from the inhalation of fibres suspended in ambient air. Resulting disease was influenced by the type and dimension of the fibres, (Craighead and Mossman, 1982). For certain types of interactions, such as haemolytic activity, the degree of reactivity depends primarily on the charge of the fibre (Light and Wei, 1977).

Dimensional characteristics have also been shown to be important in activating cellular responses such as phagocytosis (Craighead and Mossman, 1982). The diameter of the fibres is important in determining their deposition in the respiratory tract. Thin fibres are carried peripherally into the parenchyma of the lung where they lodge in the terminal airways (Mossman *et al*, 1983). Chrysotile fibres, with their characteristic curled configuration tend to be deposited more proximally than the needle like amphiboles which are transported more readily to the periphery of the lung (Parkes, 1973). Furthermore, animal experiments have

demonstrated that the longer the fibres, the more fibrogenic and carcinogenic they are like to be (Davis *et al*, 1986_a, 1989; Wagner *et al*, 1984).

2. Biological effect of asbestos

2.1. Asbestos related diseases

2.1.1. Historical aspects

The first case of asbestos-associated lung disease in the UK was noted in 1900. In 1935, the first cases of asbestosis and carcinoma of the bronchus were described by Lynch and Smith and by Gloyne, but it was not until 1955 that Doll showed the true significance of this association. Further evidence of the increased risk of bronchial carcinoma in asbestos workers was provided by Newhouse (1969). In the succeeding years, this association has been amply confirmed in many countries (Jones, 1979).

Mesothelioma is a cancer affecting the lining membrane of the pleura or peritoneum. Wagner *et al* (1960), in south Africa, first demonstrated convincingly that individuals with mesotheliomas had been exposed to crocidolite occupationally or environmentally. By the mid-1960s, this association had been firmly established (Elmes and Wade, 1965). Further pleural pathological entities associated with asbestos dust exposure were described by Kiviluoto (1960) and Meurman (1966), such as pleural plaques and thickening of the parietal pleura.

2.1.2. Effect of asbestos in the lungs

The falling speed of fibrous dusts in air is the critical parameter and is dependent on their diameter rather than on their length (Timbrell *et al*, 1970_a). Following inhalation of dust-laden air, most large asbestos fibres (10 μm or larger in diameter) are trapped in the mucus and hair of the nasal passages. Fibres of smaller size may enter the bronchial tree; most particles which deposit there are cleared by the mucociliary process, particularly if they are larger than 5 μm in diameter. Smaller fibres (particularly if less than 3 μm in diameter) which are of respirable size may enter the terminal bronchioles and alveoli. It is these smaller fibres, particularly those with a diameter of less than 1 μm , which are considered to be the most significant in inducing biological effects in man (Buchanan, 1979).

A fibre which is relatively straight will tend to align itself with its major axis lying in the direction of air flow. A curved fibre, predominantly a fibre of the chrysotile type, shows no such preferred axial orientation (Parkes, 1973). There is therefore a greater possibility of chrysotile fibres coming into contact with the wall of the airways and of them being removed by the mucociliary escalator, than would be the case with amphibole fibres. Amphibole fibres are

therefore more likely to reach the periphery of the lung (Jones, 1979).

Pulmonary alveolar macrophages are highly active in their ability to phagocytose a variety of foreign particles. Alveolar macrophages have receptors on their surface for IgG and C₃b, which mediate phagocytosis of foreign substances that have been trapped in immune complexes (Daughaday and Douglas, 1976). Ingestion of particles is in general beneficial to the host. However, ingestion frequently leads to release of various enzymes and oxygen metabolites, which can be injurious to surrounding cells and tissue. Ingestion of asbestos which is a nondigestible substance may result in a macrophage that chronically continues to release these potentially injurious substances (Musson and Henson, 1984).

In many instances, asbestos bodies may be formed as the results of reaction of the lung cells and asbestos fibres. Asbestos bodies are usually rod-shaped structures with clubbed ends, often beaded along their length and yellow to brown in colour. The coating consists of ferritin granules and an amorphous material, probably protein (Gaensler and Addington, 1969). Suzuki and Churg (1969) believed that coating is an intracellular process and follows the engulfing of particles by macrophages to which they adhere. Although all fibre types may become coated in the laboratory animal, in man the amphibole fibre is found more frequently as the core of a ferruginous body than the chrysotile fibre (Becklake, 1976). Over the years, they tend to break down, releasing pieces of fibre and the lung tissue is gradually destroyed and replaced with scar tissue. In some cases, the changes are particularly marked in the pleura, which becomes thickened (Buchanan, 1979).

Following the inhalation of asbestos dust, the following possibilities may occur:

1. The majority of the dust is removed from the tracheobronchial tree by exhalation, the mucociliary escalator and swallowing.
2. Asbestos retained in the lung enters the bronchioles and alveoli. Some fibres may penetrate bronchiolar and alveolar walls to remain in the interstitial tissue, some may migrate to the pleural surface or enter lymphatic vessels.
3. A high proportion of inhaled asbestos fibres that are retained in the lung become asbestos bodies.

2.1.3. Pathological changes associated with asbestos exposure

The main pathological changes associated with the inhalation of asbestos dust may be summarized as asbestosis, bronchial carcinoma, mesothelioma and pleural plaques.

2.1.3.1. Asbestosis

Asbestosis is a lung fibrosis of the professional asbestos worker in that its onset is generally dependent on prolonged and relatively heavy exposures to asbestos dust. Asbestosis is associated with all commercially used types of asbestos and tends to progress even although exposure may have been ended (Buchanan, 1979).

Asbestos exposure is also associated with a variety of diseases affecting multiple organ systems, such as the gastrointestinal system (Becklake, 1976). Immunologic abnormalities have also been described in asbestosis patients (Miller and Kagan, 1981; Lawrence *et al*, 1982).

The mechanisms whereby asbestos fibres exert their fibrogenic effects in the lungs are not fully understood. However, alveolar macrophages are likely to play a central role. Macrophages may mediate asbestos-induced fibrosis by releasing lysosomal enzymes (Kagan, 1985_b), free radicals (Kandaswami *et al*, 1988), prostaglandins, leukotrienes, and macrophage derived growth factor for fibroblasts (Lemaire *et al*, 1986_b) as well as IL-1 (Beckert *et al*, 1989) and TNF (Driscoll *et al*, 1990).

During asbestosis, there is almost invariably some fibrotic thickening of the visceral pleura, predominantly in the basal region. Fusion of the visceral and parietal layers of pleura may be present (Jones, 1979).

2.1.3.2. Bronchial carcinoma

Workers exposed to asbestos have an increased risk of developing bronchial carcinoma. This risk appears to be related to the exposure dose as well as the type of asbestos, being greater among workers exposed to crocidolite and amosite than among those exposed to chrysotile or anthophyllite (Seaton, 1984). More than the expected number of cases of alimentary tract carcinoma have also been reported in asbestos workers (Harington, 1981).

2.1.3.3. Mesothelioma

It has been generally accepted that primary serosal tumours of the pleural and peritoneum (mesothelioma) exist as a distinct pathological entity. Characteristically, the dense white or grey-yellow tumour is usually found initially in one pleural cavity, and both the visceral and parietal layers of the pleura are involved (Jones, 1979). Wagner and his colleagues (1960) first reported 33 cases of the rare pleural or peritoneal mesothelioma from the North-West Cape of South Africa. A variety of animal studies by intrapleural injection and inhalation of different kinds of asbestos confirmed the pathogenesis of asbestos in induction of mesothelioma (Wagner *et al*, 1970, 1974; Davis 1970; Stanton and Wrench, 1972).

2.1.3.4. Pleural plaques

Plaques are pleural lesions made up of hyalinized fibrous tissue located on the parietal pleura of the thorax, diaphragm, mediastinum, and pericardium (Craighead and Mossman, 1982). They appear as yellow-white areas of patchy thickening, projecting slightly above the contour of the parietal pleura. Pleural plaque formation does not seem to depend on particularly heavy exposure to asbestos dust, and any of the commercially used types of asbestos can lead to this pleural change (Jones, 1979). In general term, the formation of pleural plaques indicates exposure to asbestos, but the plaques are not harmful.

2.2. Mechanisms of asbestos-induced lung damage

The pathogenic framework common to all interstitial lung diseases consists of a stimulus, activation of immune effector cells, and the development of an alveolitis. The outcome of this alveolitis may be resolution either with or without scarring or the development of pulmonary fibrosis. In asbestos-related pathologic changes, the asbestos fibre is the responsible stimulus.

2.2.1. Role of alveolitis in interstitial lung diseases

The interstitial lung diseases including asbestosis are a group of chronic disorders that involve the entire parenchyma as well as the alveolar interstitium (Crystal *et al*, 1981). The central pathogenic mechanisms operative in these diseases are those that relate to maintenance of alveolitis. The alveolitis of the interstitial lung disorders is characterized by an increase and shift in the relative proportions of inflammatory and immune effector cells present, such as activated macrophages and PMN (Fulmer and Crystal, 1979). This shift is thought to be a key to the pathogenesis of these disorders through the release of inflammatory and immune mediators by effector cells, which have the potential to markedly derange the alveolar structure (Crystal *et al*, 1981).

In the acute lung disorders, the amount of inflammation does not significantly damage the alveolar structure. In contrast, the chronic alveolitis of the interstitial lung disorders, such as asbestos-induced lung disorders, presents a continual burden of immunoglobulins (Bignon *et al*, 1978), oxidants (Case *et al*, 1986) and proteolytic enzymes (Begin *et al*; 1983) that overcome the protective measures of the lower respiratory tract.

Alveolar macrophages from asbestos-exposed sheep were found to produce a chemotactic factor which attracted peripheral blood neutrophils *in vitro*. The chemoattractant attracted neutrophils more efficiently when it was obtained from higher-exposure than from lower-exposure animals. Bronchoalveolar lavage fluids from the animals also showed chemotactic activity for peripheral blood

neutrophils. Supernatants of control macrophages exposed to asbestos *in vitro* were also chemotactic for PMN (Rola-Pleszczynski *et al*, 1984). In a hamster study, bronchoalveolar lavage revealed a persistent neutrophil increase that began within 24 h of asbestos treatment; this was associated with the spontaneous release of neutrophil chemotactic activity by cultured alveolar macrophages (Glassroth *et al*, 1984). Schoenberger *et al* (1982) confirmed that macrophage-derived neutrophil chemotactic factor plays a role in modulation of PMN migration to the lung after acute asbestos exposure. Similar results were shown by Hayes *et al* (1990) that asbestos induced the release of a human alveolar macrophage-derived neutrophil chemotactic factor.

Kagan *et al* (1986) demonstrated that macrophage culture supernatants obtained from both crocidolite-exposed and chrysotile-exposed rats showed significantly greater chemoattractant activity towards rat fibroblasts than similar culture supernatants from sham-exposed animals.

2.2.2. Effects of asbestos on bronchoalveolar lavage profile

In an observation by Gellert *et al* (1985), 46% of patients with asbestosis showed an increase in the percentage of neutrophils and 29% showed an increased percentage of lymphocytes. Begin *et al* (1983) observed that when individuals were exposed to asbestos, neutrophils were found in the bronchoalveolar lavage (BAL) fluid. Xaubet *et al* (1986) and Robinson *et al* (1986) have described increased levels of eosinophils in the BAL of asbestos-exposed individuals.

When sheep were instilled with UICC Canadian chrysotile fibres at low-dose, there was a significantly higher yield in the total BAL cells due to a marked increase in the macrophage and a small quantitative rise in the lymphocyte populations (Begin *et al*, 1981). The anatomically isolated sheep tracheal lobe was exposed to chrysotile fibres and BAL analysis documented persistent increases in macrophages and neutrophils. Lung histology revealed a macrophage and neutrophil peribronchiolitis and alveolitis (Begin *et al*, 1983).

Rats inhaling chrysotile asbestos also showed a macrophage/ neutrophil alveolitis (Donaldson *et al*, 1988_c). Bozelka *et al* (1983) described an increase in the total cell number and numbers of neutrophils in mice inhaling chrysotile asbestos. Adamson and Bowden (1987_b) also confirmed a neutrophil alveolitis following the instillation of a long fibre sample of amosite using mouse model. The use of a short sample of the same mineral was only associated with a transient, resolving inflammatory response in BAL (Adamson and Bowden, 1987_a).

Rola-Pleszczynski *et al* (1981) demonstrated that after receiving monthly intratracheal instillation of chrysotile asbestos, sheep BAL leukocytes were found

to have enhanced proliferative activity in response to concanavalin A, pokeweed mitogen, and staphylococcal lysate.

Asbestos exposure is also associated with changes in the immune system. Bignon *et al* (1978) demonstrated that patients with asbestosis had higher average concentrations of albumin, IgG, IgA and α_1 anti-trypsin in their BAL fluid than did subjects with asbestos exposure with or without other asbestos related diseases. Guzman *et al* (1986) showed activated T-cells and increased helper/suppressor ratios in BAL fluid of asbestos-exposed individuals. Rat alveolar macrophages exposed to asbestos have increased numbers of surface IgG-Fc and C_3 receptors (Tetley *et al*, 1976). Begin *et al* (1983) found an increase in γ -globulins in BAL of sheep following exposure to chrysotile asbestos.

Asbestos exposure also affects the metabolism of extracellular matrix in the lung. One investigation (Begin *et al*, 1986) indicated that the levels of fibronectin and procollagen 3 in BAL fluid were significantly elevated in the asbestos-exposed sheep, as well as in those with asbestos-associated alveolitis or asbestosis.

2.2.3. The change of enzyme in BAL caused by asbestos

Begin *et al* (1983) showed that proteins and enzymes in the BAL fluid increased significantly in the high-dose group of chrysotile-exposed sheep. Tetley *et al* (1976) demonstrated that chrysotile induces elevated levels of lysosomal enzyme in both the whole lung and free cell population. Significantly higher β -glucuronidase and lactate dehydrogenase activity was found in BAL fluids from chrysotile and crocidolite asbestos-exposed groups compared to controls (Kagan *et al*, 1983_a; Begin *et al*, 1983). An inhalation exposure of rats to chrysotile asbestos also resulted in increased levels of free enzymes and total protein (Donaldson *et al*, 1988_b). *In vitro* pulmonary alveolar macrophages exposed to asbestos produce lysosomal enzymes (Schoenberger *et al*, 1982).

Davies (1980) reported that asbestos was cytotoxic towards the macrophage releasing both lactate dehydrogenase and β -glucuronidase and asbestos containing long fibres caused a significantly higher release of the lysosomal β -glucuronidase. The observation of Hamilton (1980) indicated that intraperitoneal injection of chrysotile fibres in mice elicited a cellular exudate. The macrophages in the exudate produced high levels of the neutral protease, plasminogen activator. White and Kuhn (1980) showed that peritoneal exudative macrophages increased their secretion of elastase in response to phagocytosis of chrysotile and crocidolite.

2.2.4. Oxidant metabolism during alveolitis caused by asbestos

The exposure of hamster and rat alveolar macrophages *in vitro* to nontoxic concentrations of crocidolite asbestos caused a significant increase in both release

of superoxide from rat macrophages and enhancement of zymosan-triggered superoxide from hamster macrophages (Hansen and Mossman, 1987). An inhalation study of sheep exposed repeatedly to UICC chrysotile B asbestos and quartz indicated that lung-inflammatory cells from sheep exposed to asbestos released much higher levels of superoxide in the presence of phorbol myristate acetate than did cells of unexposed sheep (Cantin *et al*, 1988).

Goodglick *et al* (1989) showed that after mouse peritoneal exposure to crocidolite asbestos the macrophages produce lipid peroxidation. Case *et al* (1986) reported that the presence of crocidolite and chrysotile evoked significant rises in O_2^- release by alveolar macrophages.

Roney and Holian (1989) investigated the possible mechanism of asbestos-stimulated superoxide anion production and showed that the mechanism of guinea pig alveolar macrophage stimulation by chrysotile is consistent with a mechanism which is similar to that used by agonists such as *N*-formyl-Nle-Leu-Phe resulting in stimulated phosphatidylinositol turnover, calcium mobilization, and activation of protein kinase C. Scheule and Holian (1989) in another study demonstrated that immunoglobulin G specifically enhances chrysotile asbestos-stimulated superoxide anion production by guinea pig alveolar macrophages. It was suggested that asbestos may stimulate macrophages by crosslinking cell-surface immunoglobulin Fc receptors (Scheule and Holian, 1989).

2.2.5. Fibrogenicity of asbestos

Wagner *et al* (1974) reported that in a animal inhalation study, amosite invariably gave the least fibrosis and Canadian chrysotile the most. Crocidolite and Rhodesian chrysotile were intermediate. The animal inhalation studies by Davis *et al* (1978) showed that chrysotile dust caused far more lung fibrosis than crocidolite or amosite when dust clouds contained roughly the same number of fibres. Johnson (1987) found that crocidolite appears to be weakly fibrogenic, and other factors may be needed to produce the marked lesions seen in human asbestosis.

In a study comparing inhaled asbestos with other dusts, Lee *et al* (1981) found that asbestos was the most potent fibrogenic agent with more than 10 times more fibrogenicity than fibreglass particles.

Chang *et al* (1988) demonstrated that a brief inhalation exposure to chrysotile asbestos caused a rapid response that involves an influx of macrophages to the first alveolar duct bifurcations and alterations in the alveolar epithelium. Johnson (1987) also found a significant increase in the numbers of rat interstitial and alveolar macrophages after asbestos inhalation. Polymorphs appeared in the

interstitium at airway bifurcations, prior to their appearance in the alveolar space. These bifurcations were revealed to be the initial sites of cell damage and collagen deposition. Chang *et al* (1988) suggested that these acute structural changes are followed by a progressive response manifested by increased numbers of interstitial cells and localized interstitial fibrosis.

2.2.6. Carcinogenesis of asbestos

Workers exposed to asbestos have an increased risk of developing bronchial carcinoma (Harington, 1981) and the risk appears to be related to the exposed dose as well as to the type of asbestos, being greater among workers exposed to crocidolite and amosite than among those exposed to chrysotile or anthophyllite (Seaton, 1984). Many studies have also shown an increased risk of other neoplasms in asbestos workers (Liddell, 1981).

Evidence exists that asbestos is a complete carcinogen, an initiator and a promoter (Barrett *et al*, 1989). Multiple mechanisms for the carcinogenic effects of asbestos have been suggested: asbestos induces chromosomal mutations, such as chromosomal changes in rat pleural mesothelial cells (Wang *et al*, 1987), sister chromatid exchange in human fibroblast and lymphoblastoid cells *in vitro* (Casey, 1983) and in circulating lymphocytes of asbestos workers (Rom *et al*, 1983). Asbestos also induces transformation of cells in culture, such as morphological and neoplastic transformation of Syrian hamster embryo cells induced by asbestos (Barrett *et al*, 1989).

2.2.7. Localisation of asbestos fibres after inhalation

Holt *et al* (1965) found in a short term Guinea pig inhalation experiment that dust reached the terminal bronchioles after a few days and trace amounts entered the alveoli. A bronchiolitis with damage to the surface epithelium followed, and the lumen became filled with cellular debris and dust-laden macrophages. There was later extension of the inflammatory infiltrate from the bronchioles into the lung, with thickening of alveolar walls and spreading fibrosis. The tracheal lymph nodes contained dust-laden macrophages in which asbestos fibres and bodies were identified.

After rats were exposed to aerosolized chrysotile, Pinkerton *et al* (1984) found that macrophages and alveolar epithelial cells contain significant amounts of asbestos and are associated with histological changes indicating marked epithelial injury. With continued exposure to asbestos, increased amounts of fibres are also localized in the lung interstitium and are associated with a progressive interstitial fibrotic reaction. Following cessation of exposure, macrophages and epithelial cells are cleared of fibres and resolve toward normal proportions. However, significant

clearance of fibres from the lung interstitium does not occur after cessation of exposure and there is a continuing process of fibrogenesis.

It was demonstrated by Pinkerton *et al* (1989) that exposure to low levels of ozone resulted in enhanced rat pulmonary retention of inhaled asbestos and implied that ambient levels of ozone can impair clearance of asbestos from the lungs.

Asbestos may also reach the pleura to cause pleural pathologic changes (Sebastien *et al*, 1979) after inhalation exposure (Section 3.4).

2.2.8. Asbestos size in pathogenesis

The historical aspects of studies on the role of fibre size in asbestos pathology has been reviewed by Davis (1979), these have emphasized the pathogenicity of long thin fibres compared to short fibres which are much less active.

A major part of the pathogenicity of mineral fibres is attributed to physical shape. The most dangerous fibres studied are erionite, which has a high length to diameter ratio, and crocidolite, which is also long and thin (Hillerdal, 1984). Hillerdal (1985) suggested that the fibrous structure is the cause of the pathological reactions. Consequently, any fibre with the critical minimal diameter (0.2 μm or less) and length (3–5 μm or more) is presumably dangerous and has been proven so in animal experiments. It was thought (Stanton *et al*, 1981; Hirsch and Bignon, 1985) that fibres less than 1.5 μm in diameter and greater than 8 μm in length cause cancer irrespective of their physicochemical nature simply because they are fibres.

Pott and Muhle (1983) suggested that a fibre with a diameter of 0.1 μm possesses a maximal carcinogenic potency. When the diameter increases the carcinogenic potency is reduced and nearly reaches the zero point at 2 μm . With regard to the length, the carcinogenic potency is beginning at about 2 μm and increases with increasing length.

A study in rats with a single intratracheal instillation of chrysotile asbestos by Lemaire *et al* (1985_a) showed that long chrysotile B has significant pathological effects on the lung. The lesions were localized in and around terminal bronchioles and consisted of inflammatory cells, fibroblasts and collagen deposition with distorted and obstructed small airways. However, no fibrosis was apparent in the lungs of these animals for short chrysotile fibres.

Davis *et al* (1986_a) found that exposure to dust clouds of long amosite fibres caused deposits of granulation tissue and progressive thickening of alveolar septa, as well as pulmonary carcinomas and pleural mesotheliomas, while exposure

to short amosite fibres showed no, or much less, pathogenicity. In another long-term inhalation and intraperitoneal injection study with rats, Davis and Jones (1988_b) confirmed that rats treated with long-fibre chrysotile developed 6 times more advanced interstitial fibrosis and 3 times more pulmonary tumours than animals treated with short-fibre chrysotile. In addition, following intraperitoneal injection, long-fibre dust produced more mesotheliomas than the short-fibre. Adamson *et al* (1987_a) demonstrated that after short crocidolite fibres were instilled into the lungs of mice, only a high dose of short fibres produces minimal lung injury and fibrosis. However, same treatment of long crocidolite fibres resulted in a severe fibrotic reaction (Adamson *et al*, 1987_b).

Lemaire (1985_b) found that there was longer increase in inflammatory cells in animals treated with long chrysotile than with short chrysotile. Differential cellular analysis indicated that after injection of long chrysotile fibres, PMN and multinucleated cells (MGC) were found in lavage fluid and most of these cells were binucleated. By contrast, exposure to very short chrysotile fibres caused only a very transient influx of PMN. Many of MGC had 3 or more nuclei. Donaldson *et al* (1989) have also demonstrated the high tissue injuring potential of long fibres after intraperitoneal deposition while a short fibre sample was virtually inactive in these respects.

Davis *et al* (1986) showed that after dust inhalation, one third of animals treated with long amosite fibre dust developed mesotheliomas but there was none in animals treated with short fibre amosite. Intraperitoneal injection also confirmed the greater carcinogenesis of the long fibre amosite than that of short fibre dust.

It was presumed that under experimental conditions short asbestos fibres (that is less than about 10 μ m) are engulfed and removed by alveolar macrophages, but longer fibres cannot be properly ingested, in which case a number of macrophages become attached to them and release various enzymes and oxygen metabolites chronically, which can be injurious to surrounding cells and tissue (Parkes, 1973; Musson and Henson, 1984 and Davis *et al*, 1988_a). In addition, long fibre asbestos may cause injury to bronchial and bronchiolar epithelium so that it can reach the interstitium to induce macrophage-fibroblast interactions there (Adamson *et al*, 1987_a).

However, Monchaux *et al* (1981) found that oxalic and hydrochloric acid leaching dramatically decreases the carcinogenic effect of chrysotile asbestos. Evans *et al* (1983) showed that surface modification of asbestos by glutaraldehyde-coupled protein can reduce the rate of fibre/cell interaction. The effect of surface modification by effectively adding C₈ and C₁₈ hydrocarbon chains was investigated

by Brown *et al* (1990). They found that both surface silylation treatments reduced the toxicity of the asbestos and had reduced fibre/cell interaction. Furthermore, in rat intrapleural studies, the C₁₈ derivatized fibre was markedly less active in the production of mesotheliomas, however, C₈ material had the same activity as the parent material but induced the tumors earlier. These data indicates that not only size characteristics but also parameters such as chemical composition and physicochemical properties must intervene (Monchaux *et al*, 1981).

3. The pleura

The principal function of the pleura is to decrease the friction and consequently enhance the efficiency of respiratory movements (Andrews and Porter, 1973; Staub *et al*, 1985).

3.1. Structure and exchange functions of the pleura

3.1.1. Anatomy and physiology of the pleura

3.1.1.1. Mesothelium

The surface lining of the parietal and visceral pleura consists of a single layer of stretchable, polygonal, mesothelial cells being diffusely covered by microvilli (Pistolesi *et al*, 1989) (Section 3.2).

3.1.1.2. Pleural connective tissue

The connective tissue of the pleura is a complex structure or layers of structure that underlies the surface layer of mesothelial cells (Bernaudin and Fleury, 1985). Pleural connective tissue contains both dense collagenous and elastic fibres which permit it to modulate lung expansion during normal respiration and to limit lung expansion during maximum ventilatory conditions. In quantitative terms, the collagens are the major component of the pleural extracellular matrix, and type I collagen comprises a major portion of pleural connective tissue (Prockop *et al*, 1979). Elastic fibres which are comprised of two components: elastin and microfibrils, make up the layers of elastic laminae present in the pleura (Agostoni, 1972). A large number of glycoproteins are found as components of the extracellular connective tissue of the pleura. Two glycoproteins, fibronectin and laminin are likely to be present in pleura. The former may play a similar role in anchoring cells to the pleural matrix (McDonald *et al*, 1982) and the latter is known to modulate the adhesion of cells to basement membranes (Terranova *et al*, 1980). In the pleural space, proteoglycans and glycosaminoglycan hyaluronic acid are also present and may function as a lubricant (Arai *et al*, 1975; Herbert, 1986).

3.1.1.3. Anatomy of the blood and lymphatic circulation of the pleural serosa

In the human visceral pleura, the two different groups of arteries, the

pulmonary and bronchial arteries, can both potentially ensure the blood supply, but the former supplies the greatest part of the pleura. The arteries of the human parietal pleura are branches of the arteries supplying the adjacent thoracic wall (Bernaudin and Fleury, 1985).

The pleural space is faced by two very different lymphatic circulations. The parietal pleura lymphatics are the true lymphatic system of the pleura, whereas the visceral pleura lymphatics are the superficial portion of the lymphatic system of the lung. The usual direction of the lymph flow in visceral pleura is from the surface of the lung to the hilum and valves ensure a one-way flow (Bernaudin and Fleury, 1985). It is believed that the visceral pleura lymphatic circulation has no direct communication with the pleural space (Albertine *et al*, 1982).

In the parietal pleura, two lymphatic plexuses can be differentiated: one in the subpleural space and a more superficial one just beneath the mesothelial layer. The lymphatics of the parietal pleura is of major physiological significance since they play an essential role in the removal of components of the pleural fluid in the normal state as well as in the case of pleural effusion (Bernaudin and Fleury, 1985). This removal is sustained by true anatomical connections, the stomata (Section 3.1.2), between the pleural space and the lumen of lymphatics of the parietal pleura (Pistolesi *et al*, 1989).

3.1.2. Exchange across the pleura

3.1.2.1 Volume, proteins, and cells of pleural liquid

Physiological experiments strongly suggest that the pleural membrane is permeable. Additionally, the existence of stomas means that the pleural cavity is directly open to the lymphatics of the chest wall without any interposed epithelium (Wang, 1975; Pistolesi *et al*, 1989).

The pleural space is a real space, not a potential one with width being 10–20 μm . Since the microvilli of the visceral and parietal mesothelial cells are 3–5 μm long, this means that there is little or no contact between these surfaces. The normal volume of liquid in the pleural spaces is small:– 0.1–0.2 ml/Kg body weight in most mammals. The protein concentration in the pleural liquid of dogs was found to be $1.06 \pm 0.11\text{g}$ per 100 ml. The computed colloid osmotic pressure was 3.2 cm H_2O (Staub *et al*, 1985). Mesothelial cells, monocytic cells, and a few lymphocytes have been found in the pleural liquid of dogs at 2208 ± 734 per mm^3 . Pleural leukocyte studies indicated that normal lavaged pleural leukocytes are comprised mainly of macrophages, mast cells, eosinophils, PMNs and lymphocytes (Oberdoerster *et al*, 1983; Zlotnik *et al*, 1982; Pitt, 1985).

3.1.2.2. Liquid and solute exchange

The passage of water and small solutes across a mesothelial cell layer can be either transcellular or paracellular via numerous plasmalemmal vesicles in the mesothelial cells (Gil, 1983). Vesicles act as shuttle vehicles in a process of transcellular transport. The alveolar/pleural boundary is also incomplete because the pleural interstitium is continuous with the alveolar septa (Gil, 1985).

Kanazawa (1985) suggested that water, electrolytes, and small molecular substances are excreted, mainly from the parietal pleura into the pleural cavity, and are absorbed via blood capillaries in the visceral pleura after penetrating the mesothelial lining cells or through the intercellular junctions. Larger molecules, such as protein, are mainly absorbed via the lymphatics in the parietal pleura. They penetrate through the pleura along the intermesothelial junctions or are transported through the mesothelial cells (Black, 1972).

3.1.2.3. Particle exchange

The absorption of fairly large particles from the pleural cavity takes place through various routes. Using injected erythrocytes, ^{there are} at least three different pathways to remove the erythrocytes from the pleural cavity: (1) by stomata in the pleura, which are 2–6 μm openings and distribute over parietal pleura at a density of about 300–400 mm^{-2} (Albertine *et al*, 1984; Kanazawa, 1985) (2) by crevices in the mediastinal pleura which are 1–10 μm in width and 5–30 μm in length (Kanazawa, 1985) and (3) in particular, by milky spots, discernible by the naked eye as small white flecks, which are the aggregates of macrophages and specialised mesothelial cells with the distribution similar to that of stomata (Herbert, 1986). It is suggested that serosal stomas and crevices are the sewage system and milky spots the surveillance system of the serosal cavities (Kanazawa, 1985).

Transportation of particles to the pleura is presumed mainly by three routes:— (1) penetration of particles through the visceral pleura (transpleural migration). Fibres located in peripheral alveoli can cross the visceral pleura and also this translocation could be facilitated by respiratory movements, macrophage migration, and mesothelial cell exfoliation (Kanazawa, 1985).

(2) Lymphatic transportation. The lymphatics from the pulmonary alveoli are connected with the submesothelial lymphatics in the visceral pleura (Lauweryns, 1974) and there is the negative pressure in the pleural cavity (Kanazawa, 1985). In addition, during asbestos exposure, partial obstruction of the trachea may increase the negative pressure of the pleura. These factors would result in transport of asbestos fibre through the visceral pleura.

(3) Blood transportation. Milky spots would allow blood-borne particles, such as asbestos fibres, to pass through the mesothelial cells into the pleural cavity (Kanazawa *et al*, 1979) because the bloodstream is slowed down within the milky spot and the basement membrane of the blood capillaries in milky spots is less well developed and discontinuous. Many phagocytes exist in milky spots to trap the particles which may be brought into the pleural cavity (Kanazawa, 1985).

However, the controversy whether or not some particles, such as asbestos fibres, can reach the pleura space exists and the mechanisms whereby the fibres reach the pleural space are still in question (Herbert, 1986).

3.2. Pleural mesothelial cells

3.2.1. Normal mesothelial cells

The pleural mesothelial cells are single-layered stretchable cells between 1 μm and 4 μm in thickness and ranging from 16.4 to 41.9 μm in surface diameter (Cooray, 1949). They have abundant elongated "brushlike" microvilli that entrap hyaluronic acid-rich glycoprotein. The microvilli on the mesothelial cell surface are characteristically elongated and randomly oriented. They are up to 3 μm in length and approximately 0.1 μm in diameter, with the length to diameter ratio usually over 10 (Wang, 1974). The pinocytic vesicles are well developed on both luminal and basal surfaces of the mesothelial cell (Madison *et al*, 1979). Mesothelial cells on rigid substructures are flat, tend to be stable with a low turnover rate, and well-developed intercellular junctions (Wang, 1985). Apical tight (zonula occludens) and gap (nexus) junctions and well-developed desmosomes are present between human mesothelial cells (Wang, 1974). The estimated life span of the pleural mesothelial cell in the rat is 33 days (Bryks and Bertalanffy, 1971).

Whitaker *et al*, (1982_a)^{found} that the normal flattened (resting) mesothelial cells show, predominantly, the enzymes of the pentose pathway whilst cuboidal (activated) cells have, predominantly, the enzymes of the oxidative pathway.

The renewal of damaged mesothelial cells include maturation of submesothelial mesenchymal cells (Ryan *et al*, 1973); the transition of free-floating cells (monocytes) in the serous exudate (Watters and Buck, 1972) and replication of already differentiated mesothelial cells at the periphery of the wounded area or from the opposite mesothelial surface (Whitaker *et al*, 1982_b). It is postulated that exudate macrophages secrete mitogenic factor(s) which stimulate mesothelial proliferation, and initiate healing (Fotev *et al*, 1987).

Rennard *et al* (1984) hypothesized that the mesothelial cells are the major source of the pleural connective tissue. Those include collagen (Harvey and Amlot, 1983; Whitaker, 1977), elastin, glycoprotein and proteoglycan (Rennard *et*

al, 1984). It has been shown that mesothelial cells exert effect in fibrinolysis (Porter *et al*, 1971). Newly regenerated mesothelium has greatly enhanced fibrinolytic activity, but fibrinolytic activity is depressed by damage to mesothelial cells (Whitaker *et al*, 1982_c).

Various types of inflammatory agent, physicochemical irritation, and biochemical irritations or stimuli (endotoxin, bovin serum albumin, and microorganisms), when deposited in the pleural space, have been shown to induce a rapid desquamation of the mesothelium (Bignon and Gee, 1985).

3.2.2. The mesothelial response to asbestos

The pleura, with its mesothelial cells, is very reactive in response to inhaled asbestos by producing two specific pathologies, fibrosis and cancer (Jaurand, 1985). Asbestos has been confirmed to cause pleural mesothelioma (Section 3.4.2). Jaurand *et al* (1983_a) reported that chrysotile asbestos induces a significant increase in the number of sister chromatid exchanges of mesothelial cells.

As confirmed by Donaldson *et al* (1988_c), crocidolite asbestos produced inhibition of plasminogen dependent fibrinolysis of mesothelial cells and may thus permit formation of a fibrin scaffold which would favour fibrinogenesis. Bryks *et al* (1971) observed a marked increase in the proliferation of visceral mesothelium 5-7 days after intratracheal administration of chrysotile in the rat (Herbert, 1986). Renier *et al* (1990) reported that rat pleural mesothelial cells treated with chrysotile or crocidolite showed a significant dose-dependent increase in [³H]dThd incorporation compared to untreated cells, which indicates that asbestos produces unscheduled DNA synthesis in the cells.

Antony *et al* (1989) reported that mesothelial cells in the presence of crocidolite asbestos released chemotaxins for neutrophils which can produce proteases capable of degrading supporting matrix to increase the pleural permeability.

Additionally, inflammatory processes related to asbestos exposure can lead to the development of adhesions between the parietal and visceral pleura (Stephens and Gibbs, 1987), which may result from the disruption of the adhesive molecules that likely maintain the normal anchorage of mesothelial cells (MacDonald *et al*, 1979). With the loss of the lining of cells from a pleural surface, the exposed underlying connective tissue could serve as a substrate for attachment of the opposite pleural surface and lead to adhesion formation (Rennard *et al*, 1985).

Davis (1974) found that at an early stage after intrapleural injection

mesothelial cells became rounded and less clearly attached to one another. Later in the studies the mesothelium returned to normal, but where mesothelium covered asbestos granulomata, the cells were extremely flattened, and without surface microvilli. In some areas pores were found penetrating the mesothelial cell cytoplasm and leaving areas of connective tissue in direct contact with the pleural cavity.

3.3. Leukocytes of the pleural space

The pleural space, like the peritoneal space and other serosal surfaces, has a permanent leukocyte population. Pleural leukocyte numbers found in dog were 2208 ± 734 per mm^3 (Miserocchi and Agostoni, 1971). Lavaged leukocyte numbers were $7-13 \times 10^6$ in rat (Oberdoerster *et al*, 1983), 3.295×10^6 BALB/c mice and 1.758×10^6 in CF-1 mice respectively (Zlotnic *et al*, 1982) the differential counts indicated that pleural leukocyte population obtained from normal BALB/c mice contained: 51.3% macrophages, 47.6% lymphocytes, 0.4% eosinophils and 0.8% mast cells. Pitt (1985) reported that the cell population obtained from 3-18 months old BALB/c mice pleural space was 2.9×10^6 cells. Of these, lymphocytes comprised 70% of the total population, monocytes/macrophages 27%, PMNs less than 1% and degenerate cells 3%. The work of Oberdoerster *et al* (1983) revealed that in control pleural lavages, PMN accounted for 27- 45%, mast cells 11-22%, eosinophils 11-17% whilst others were 25- 45%.

3.3.1. Macrophages/monocytes

Most tissue macrophages derive from the circulating monocytes (Sibille and Reynolds, 1990) although some are derived from local replication (Evans *et al*, 1987). Maturation in a given tissue location confers distinctive properties on the specific macrophage type although they still retain considerable adaptive ability (Bignon and Gee, 1985). In the pleura, it would be anticipated that the same situation pertains.

A study comparing pleural macrophages to peritoneal and alveolar macrophages in mice showed that pleural and peritoneal macrophages were more similar to each other than to alveolar macrophages. Compared to alveolar macrophages, pleural macrophages migrate well on the plastic surface of the dishes, phagocytise BCG more efficiently; pleural macrophages are also capable of producing arginase whereas alveolar macrophages are not (Zlotnik *et al*, 1982).

Peritoneal macrophages have been extensively studied. They are more dependent on anaerobic glycolysis and the O_2 tension in both the peritoneal and pleural cavities is low, suggesting that a similar energy metabolism will be shown by pleural macrophages. Peritoneal macrophages induced by acute and chronic

inflammatory stimuli variably produce oxidants and generally secrete large amounts of neutral proteases such as elastase, collagenase, and plasminogen activator (Bignon and Gee, 1985).

3.3.2. Lymphocytes

Lymphocytes may influence fibrogenesis in the pleura. Human peritoneal blood T lymphocytes, activated by nonspecific mitogens or antigen, produce a nondialyzable factor that stimulates proliferation of dermal fibroblast and synthesis of collagen (Wahl *et al*, 1978). They also release fibroblast chemotactic factors (Postlethwaite *et al*, 1976). Postlethwaite and Kang (1982) found that the monocyte-derived IL-1 effects on fibroblast proliferation via the lymphocyte-derived fibroblast chemotactic factor. However, as regards the peritoneal cavity, T lymphocytes alone seem insufficient for producing a fibrogenic factor (Bignon and Gee, 1985).

Lymphocytes are present in almost every pleural effusion and tend to be particularly abundant in effusions caused by tuberculosis or neoplasia and in long-standing effusion (Herbert, 1986), indicating a role for T-cells in mediating pleural fibrosis. Since certain lymphokines are capable of activating the coagulation system, local cellular immunity may have a role in pleural inflammation via the generation of a fibrin meshwork (Pettersson, 1985). The deposition of fibrin would form a scaffold that activates fibroblast proliferation and subsequently is colonized by fibroblasts, which may lead to fibrosis finally (Bignon and Gee, 1985).

3.3.3. Eosinophils

Morphologically, the eosinophil is characterised by its content of eosinophilic granules. The granules contain four main proteins: the eosinophil cationic protein, eosinophil peroxidase, eosinophil protein X (or eosinophil derived neurotoxin), and major basic protein (Gleich and Adolphson, 1986). Besides as a potent ribonuclease, these proteins are cytotoxic and capable of inducing histamine release from mast cells and basophils and also, stimulating glycosaminoglycan production by human fibroblasts. In addition, eosinophils produce inhibitors of T lymphocyte responses and also potently produce oxygen derived toxic metabolites such as O_2^- , H_2O_2 and OH^- (Pincus *et al*, 1981) and of various lipid mediators, such as prostaglandins (Hubscher, 1975). These cells also contain proteolytic enzymes and are cytotoxic to mesothelial cells by releasing oxygen metabolites (Bignon and Gee, 1985). Since murine eosinophils were found to be able to express IL-1 mRNA when stimulated with LPS (Pozo *et al*, 1990), eosinophils may have proinflammatory activity through the production of IL-1.

By these means the eosinophils may regulate cell mediated immunological reactions and tissue injury/repair processes (Venge, 1990).

3.3.4. Mast cells

Mast cells are characterised by their metachromatic granules and by their synthesis of a number of recognized pharmacologically potent mediators. Some of these mediators, such as histamine, heparin, eosinophil chemotactic factor, platelet-activating factor and proteolytic enzymes, are produced and stored in the granules, to be released on stimulation (Lee *et al*, 1985). Others, such as prostaglandin and lymphokines, are produced when the cells are stimulated (Plaut *et al*, 1989). Besides allergy and anaphylaxis, mast cells have role in stimulation of fibroblast proliferation (Atkins *et al*, 1985) and cytotoxicity against tumour cells (Henderson *et al*, 1981). Mast cells are also involved in TNF mediated biological processes by synthesis of TNF mRNA in their cytoplasm and TNF protein in their granules (Steffen *et al*, 1989).

The presence of large numbers of mast cells in the pleural interstitium (Pinchon *et al*, 1980) is relevant to permeability because the mast cell contains many large secretory granules rich in vasoactive substances, in particular histamine, known to play a role in altering vascular permeability, during inflammatory reactions. Clearly, a local release of histamine could lead to increased extravasation of fluids (Gil, 1985).

3.3.5. Polymorphonuclear neutrophils

Polymorphonuclear neutrophils (PMNs) are specialized cells present early in any kind of inflammation. The main function of PMN is to sense, approach, and destroy invading microorganism (Lew, 1990).

PMN can release large amounts of oxygen metabolites, including O_2^- , H_2O_2 , and OH^\cdot , depending on the stimulus and on its concentration (Rosen and Klebanoff, 1979). The azurophilic and specific granules in PMN contain proteolytic enzymes. These include acid hydrolases, myeloperoxidase, lysozyme, and neutral serine proteases. Among those enzymes, neutrophil collagenase degrades type I collagen and neutrophil elastase cleaves elastin, types III and IV collagens, and other proteins, including immunoglobulins at neutral pH (Mainardi *et al*, 1980; Fick *et al*, 1984). PMN are a source of another neutral protease, plasminogen activator (Unkeless *et al*, 1974). Furthermore, neutrophils also contain acid proteases, notably chymotrypsin and cathepsins, ^{which} are needed for killing bacteria, can also act extracellularly and injure tissues as well as structural material such as hyaluronic acid, a compound present in some pleural effusions (Bignon and Gee, 1985). PMN could release cytokines, such as IL-1 (Canning and Neill, 1989), and

sometimes this release requires the presence of zymosan or prolonged periods of incubation time. They also release concomitantly one or several IL-1 inhibitors (Sibille and Reynolds, 1990).

3.4. Pleural response to asbestos

The pleura is a primary target for various reactions resulting from asbestos exposure. The main reactions are acute pleurisy, pleural plaques, and diffuse pleural fibrosis (Hillerdal, 1984), as well as mesothelioma (Wagner, 1986).

3.4.1. Pleural leukocyte response to asbestos exposure

In the study by Oberdoerster *et al* (1983), rats received an intrabronchial instillation of amosite or saline. Beginning at 62 days after instillation, lavaged cell numbers increased significantly in the amosite group compared to controls. Furthermore, the average number of peroxidase positive macrophages, young monocytic cells, was significantly greater in amosite treated animals throughout the experiment. Pitt (1985) showed that there was a significant increase in the mouse pleural cavity free cell population after 6 months inhalation of UICC crocidolite asbestos, and this increased further with prolonged exposure. Pleural macrophages showing a marked increase in number after 9 months exposure and further investigation indicated that they were a stimulated population.

Shore *et al* (1983) demonstrated that crocidolite asbestos intrapleural injection in rabbit resulted in the appearance of chemotactic activity to PMN in an exudative effusion peaked ~~at~~ 4 hr after the injection. Edwards *et al* (1984) found that after intrapleural injection with crocidolite and chrysotile asbestos, pleural lavage showed a predominance of PMN and eosinophils at 72 hours. Total macrophages were greater with crocidolite than with chrysotile, and mast cells were markedly decreased with the two types of fibres. In rats with mesotheliomas, the pleural lavages showed a 10 to 20 fold increase in cell numbers, as might be expected with many of these being tumour cells.

3.4.2. Pleural pathologic changes caused by asbestos exposure

3.4.2.1. Pleural effusion

Patients working with asbestos may develop pleural effusion and this is the commonest asbestos-related disease during the first two decades of exposure at work (Seaton *et al*, 1989). Hillerdal and Ozesmi (1987) reported that, for developing benign asbestos pleural effusion, the mean latency time from the first exposure to asbestos was 30 years, with a range of 1 to 58 years. Even a comparatively slight occupational exposure may be sufficient and the effusion can occur many years after exposure to asbestos. Preger (1978) found that asbestos-related pleural infusion was an exudate with a protein content varying

from 3.4 to 7.5 mg/100ml. Cell counts including RBC and leukocytes in the range of 5,000 to 50,000 cells/ml are usually present. The inflammatory cell content, PMN, monocytes, lymphocytes, and eosinophils are variable.

Herbert (1986) suggested that diffuse effusion may follow acute asbestos pleurisy and is probably caused by direct damage to the mesothelium. Extensive, irregular pleural calcification as well as diffuse fibrosis of the visceral pleura may be a sequel to pleural effusion (Seaton, 1984).

3.4.2.2. Pleural plaques

Pleural plaques are strongly associated with an occupational and environmental exposure to all types of asbestos and with the presence of asbestos bodies in lung tissue (Herbert, 1986). Fibrohyaline plaques locate typically on the posterolateral and inferior part of the parietal pleura and on the dome of the thoracic surface of the diaphragm (Bignon and Gee, 1985). Hillerdal (1978) believed that pleural plaques generally are radiologically visible only after a long period, at least 15 years from initial exposure to asbestos.

Hillerdal (1980) suggested that pleural plaques can be regarded as a mild reaction to fibres that have reached the pleura by piercing lung tissue under the influence of mechanical forces within the lung and lymphatic drainage in the parietal pleura, where they possibly cause traumatic micro-haemorrhages and fibrin deposition (Parkes, 1982).

Asbestos fibre counts of the lungs of patients with pleural plaques showed a relative predominance of commercial amphiboles (crocidolite and amosite) in contrast with the predominance of chrysotile fibres found in controls (Churg, 1982; Seaton, 1984). However, the presence of plaques does not correlate with the degree of pulmonary asbestosis (Parkes, 1982).

Sahn and Antony (1984) found that following intrapleural asbestos, treatment of animals with nitrogen-mustard, which causes neutropenia, and asbestos resulted in less total inflammatory cells than in asbestos alone treated rabbits. At autopsy, nitrogen-mustard/asbestos-treated animals developed marked fibrosis in the pleural space instead of pleural plaques observed in asbestos treated animal. They suggested that the pleural macrophage is important in localizing the asbestos fibre and in the ultimate formation of the pleural plaque. Without a critical number of macrophages, plaques are not formed and instead, diffuse pleural fibrosis results.

3.4.2.3. Pleural thickening and diffuse pleural fibrosis

Pleural thickening is a common result of asbestos exposure and appears accompanying plaques on the parietal pleura. Diffuse pleural thickening affects

both parietal and visceral pleura (Hillerdal, 1981). Bignon and Gee (1985) reported that a diffuse or focal fibrous thickening of visceral pleura is commonly seen as an accompaniment of asbestos alveolitis. Klerk *et al* (1989) investigated a population of crocidolite mine workers and found that the level of total cumulative exposure to crocidolite increased the rate of onset of pleural thickening.

Pleural fibrosis is usually the sequel to an unabsorbed pleural effusion. Any cause of pleural exudate may be responsible for the subsequent development of pleural fibrosis (Seaton *et al*, 1989). A study on diffuse pleural fibrosis (Stephens and Gibbs, 1987) indicated that all of the cases had extensive visceral pleural fibrosis and extensive areas of adhesions.

Herbert (1986) believed that the mechanisms concerned in mesothelial injury and repair are important in the pathogenesis of pleural fibrosis. The fibrinolytic power of serosal surfaces, with respect to the fibrinolytic activity of mesothelial cells, is closely related to whether fibrous exudate is absorbed (Whitaker *et al*, 1982_c). Ryan *et al* (1973) showed that the fibrinolytic activity of mesothelial cells was depressed by damage to the cells. In an *in vitro* rat study, Donaldson *et al* (1988_c) demonstrated that the fibrinolytic activity of mesothelial cells was inhibited by co-culture of the cells with crocidolite asbestos. Therefore, the inhibition of the fibrinolytic activity of mesothelial cells by exposure to asbestos may partly explain the pathogenesis of pleural fibrosis. Good *et al* (1979) reported that intrapleural urokinase inhibited the development of pleural fibrosis in rabbits treated with high-dose tetracycline. This suggests that the pathogenesis of pleural fibrosis caused by asbestos is mediated through fibrin deposition.

3.4.2.4. Pleural mesothelioma

Wagner and his colleagues (1960) first reported 33 cases of the rare pleural or peritoneal mesothelioma from the North-West Cape of South Africa.

Mesothelioma may result from low exposure levels, particularly with crocidolite dust, although other types of asbestos can also cause this variety of cancer (Wagner *et al*, 1980). The exposure may not only have been brief but may also have occurred many years (20–30 years) previous to the appearance of the mesothelioma (Buchanan, 1979; Jones *et al*, 1977).

Berry and Newhouse (1983) showed convincingly that there is a gradient from crocidolite > amosite > chrysotile in the potential to cause mesothelioma. Wagner (1986) also presented evidence that crocidolite carries the greatest risk of subsequent mesothelioma formation. Other mineral fibres, if straight and having the defined length and diameter, may have the potential for causing malignant

transformation of the mesothelial membranes. However, on the vexed question of dose-response relationship in mesothelioma formation, it is too early to draw firm conclusions (Jones, 1979).

Wagner *et al* (1962) was the first to induce mesotheliomas in rats after the intrapleural inoculation of asbestos fibres. Later, many studies confirmed that after intrapleural and intraperitoneal exposure, chrysotile, crocidolite and amosite asbestos induced mesotheliomas in rat, rabbit and Syrian golden hamsters (Reeves *et al*, 1971; Stanton and Wrench, 1972). Smith and Hubert (1974) as well as Bolton (1983) showed that the mesothelioma response to chrysotile, amosite, and crocidolite was dose related.

The comparison between different kinds of asbestos revealed that after intraperitoneal injection, chrysotile samples tended to be more carcinogenic than the amosite and crocidolite preparations and these fibres produced a graded dose response for mesothelioma development with no effective minimum threshold (Bolton *et al*, 1982; 1983 and Davis *et al*, 1986). In the studies of Wagner and Berry (1969), amosite was found to produce fewer tumors than did chrysotile and crocidolite and needed a longer induction period.

Pott *et al* (1980) reported that rabbits developed mesothelioma and fibrosarcoma six years after intraperitoneal injection of UICC chrysotile asbestos and found that fibrosis is not an absolute requirement for the development of a fibre-induced mesothelioma.

Asbestos was also compared with other fibres in rat intrapleural inoculation study. Wagner *et al* (1973) reported that very small number of mesotheliomas were found in groups treated with synthetic aluminium silicate fibres, barium sulfate, glass powder, and aluminium oxide. No mesothelioma was observed in rats injected with glass fibres, or in control rats. However, Stanton (1974) developed mesothelioma in rats after intrapleural glass fibres.

As the more realistic experiments, rats were exposed by inhalation to the UICC asbestos for periods between 1 day and 2 years (Wagner *et al*; 1974). Mesothelioma were obtained with crocidolite, chrysotile, and amosite. Two such tumors occurred after only 1-day's exposure to crocidolite and amosite.

The combined effect of asbestos given by intratracheal instillation and cigarette smoke in dogs was investigated by Humphrey *et al* (1981). The results showed that there was not a clear synergistic effect between asbestos and cigarette smoke in induction of mesotheliomas. Davis and his colleagues (1991) reported that when rats were exposed to dust clouds consisting of a mixture of amosite or chrysotile asbestos with quartz or titanium dioxide, the incidence of mesotheliomas

increased and tended to occur earlier compared to asbestos alone. There was evidence of increased transport of fibres across the visceral pleural surface.

Besides asbestos, erionite, a fibrous zeolite differing markedly from asbestos in many physicochemical characteristics (Coffin *et al*, 1989), was recognized to cause the high incidence of mesotheliomas in the Anatolian region in Turkey (Baris *et al*, 1978). Wagner *et al* (1985) confirmed in rat inhalation studies that erionite produced much higher incidence of mesothelioma than crocidolite did.

In addition, ceramic fibres, a type of synthetic, amorphous and glassy fibre, were found to induce pleural mesothelioma on hamsters and rats in high-dose inhalation studies (ECFIA, 1991).

3.4.3. Translocation of asbestos fibre into the pleura

It is controversial whether asbestos depositing in airspaces following inhalation will be transferred into the pleural space. A kinetic study was performed by Viallat *et al* (1986) with rats injected intratracheally with chrysotile asbestos. The overall number of fibres present in the pleural cavity ranged from 3.6×10^4 on day 14 to 12.8×10^4 on day 21. Most fibres retrieved were short fibrils, with mean lengths ranging from 0.44 to 1.32 microns, and about 0.3 microns in diameter. They proposed the occurrence of an early blood migration.

Asbestos fibres in pleural and parenchymal tissues were measured in humans (Sebastien *et al*, 1980). In parietal pleura there were 27, 64 and 71 percent significantly positive samples from the normal location, normal plaques, and diaphragmatic plaques, respectively and they were mostly chrysotile type fibres. There was an increased short fibre frequency and decreased long fibre frequency from lung to pleura. Sebastien *et al* (1979) also found that almost all the fibres in the pleura were chrysotile fibres, but no fibre type predominated in parenchymal samples.

With regard to the mechanisms of translocation of asbestos fibres into the pleura, Holt (1983) demonstrated the migration of macrophages carrying asbestos fibres through the parenchyma of the lung towards the pleura. An inhalation study by Morgan *et al* (1977) with radiolabelled anthophyllite exhibited the accumulation of fibres within a few months in mediastinal lymph nodes which might serve as a reservoir or as a filter between the lung parenchyma and the pleura or subpleural foci. Kanazawa *et al* (1979) demonstrated in mice that after subcutaneous and intravenous injections of asbestos significant numbers of fibres were found in the milky spots of the serous cavities. A study in rats intratracheally injected with asbestos indicated two phases of asbestos translocation into the pleura also in favor of asbestos blood transportation (Viallat *et al*, 1986).

However, some experiments had opposite findings. A study on rats indicated that after intrabronchial instillation of amosite, no fibres were detected in pleural lavage samples over the experimental period (Oberdoerster *et al*, 1983). In a study on guinea pigs intratracheally instilled with amosite, Dodson and Ford (1985) showed that in all time periods up to 3 months postexposure, asbestos fibres were never found within the pleura or subpleural regions, although some fibres were observed close to the pleura. However, pleural changes occurred in the absence of direct fibre contact, including mesothelial cell pleomorphic changes, proliferation and increased vacuolization.

4. Leukocyte cytokines (monokines)

The cytokines are extracellular signalling proteins secreted by specific cells (Goustin *et al*, 1986) and these molecules have, as their primary function, the ability to modify the behaviour of other closely adjacent cells. Each cytokine acts on a particular subset of neighboring cells by interacting with specific high affinity receptors (Larrick and Kunkel, 1988). These receptors are glycoproteins, integrally located within the cell membrane that have extracellular, transmembrane, and intracellular domains. The extracellular portion of the receptor binds the cytokine ligand; the intracellular portion is involved in initiating a signalling cascade (Kelley, 1990).

It has become clear that the actions of combinations of cytokines are complex and cannot be predicted (Kelley, 1990). The complex interactions between cytokines has led to the concept that tissue homeostatic mechanisms are controlled by cytokine cascades and networks rather than by individual cytokines (Kohase *et al*, 1987).

4.1. Interleukin 1

Interleukin-1 (IL-1) is the term given to two polypeptides (IL-1 α and β) that possess a wide spectrum of immunological and nonimmunological activities. Although both forms of IL-1 are distinct gene products, they recognize the same receptor (Larrick and Kunkel, 1988). In general, there does not seem to be any difference in the spectrum of biological activities of either form (Dinarello, 1988).

4.1.1. Cellular sources of IL-1

A variety of cells have been shown to produce IL-1, including mononuclear phagocytes, B-cell, epidermal cell, glial cells and PMN (Canning and Neill, 1989); the major producers, however, appear to be monocytes/ macrophages (Martin and Resch, 1988), these include circulating blood monocytes, pulmonary alveolar macrophages, hepatic Kupffer's cells, peritoneal macrophages and splenic macrophages (Dinarello, 1984).

Although small amounts of IL-1 may be released spontaneously, most normal cells produce IL-1 only in response to exogenous stimulants (Martin and Resch, 1988), such as infection, microbial toxins, inflammatory agents, products of activated lymphocytes, complement, and clotting components (Dinarelli, 1989). Oppenheim *et al* (1989) suggested that constitutive production of IL-1 by normal cells can probably be attributed to prior stimulation *in vivo* or, alternatively, due to the presence of a contaminating stimulant *in vitro* such as the ubiquitous endotoxin.

Lonnemann *et al* (1989) reported that human mononuclear cells produced both cell-associated and secreted IL-1 α and IL-1 β *in vitro* in response to lipopolysaccharide (LPS). Wewers *et al* (1984) found that alveolar macrophages were at least 1,000 times less sensitive to LPS compared with blood monocytes in terms of IL-1 release. This limitation was thought to be linked to the maturational state of the mononuclear phagocyte. The study of Elias *et al* (1985) also showed that LPS stimulated blood monocytes elaborated more IL-1 than stimulated alveolar macrophages. In addition, denser alveolar macrophages and blood monocytes elaborated more IL-1 than less dense alveolar macrophages and monocytes. When monocytes matured *in vitro*, they lost their ability to elaborate IL-1, and became less dense. Fuchs *et al* (1988) found that after intravenously administered bacillus Calmette-Guerin, lavaged alveolar macrophages containing increased percentages of higher density cells and elaborated increased IL-1.

PMN have been demonstrated to be capable of synthesizing IL-1. Tiku *et al* (1986_a) found that normal human PMN can be stimulated by particulate agents such as zymosan and soluble agents such as phorbol myristic acetate to produce IL-1. Canning and Neill (1989) reported that the supernatants of purified peripheral bovine blood PMN cultured in the presence of opsonized zymosan particles exhibited IL-1 activity. Kusaka and Donaldson (1990_b) showed that IL-1 like activity was spontaneously produced by neutrophils obtained from experimentally inflamed rat lung at high levels, but it could still be enhanced by stimulation with endotoxin *in vitro*.

Besides monocytes, macrophages and PMN, murine eosinophils were also reported to produce IL-1 (Pozo *et al*, 1990) when they were stimulated with LPS.

4.1.2. Modulation of IL-1 production

The production of IL-1 is regulated by many factors, such as interferon and TNF. Incubation of human blood monocytes with interferon γ or α prevented the loss of IL-1 secretory potential in response to endotoxin during the differentiation from monocytes to macrophages and interferon γ was more potent

than interferon α (Haq and Maca, 1986). Gerrard *et al* (1987) reported that interferon α directly induced IL-1 secretion by monocytes whereas interferon γ greatly enhanced IL-1 secretion by priming monocytes to be more sensitive to IL-1-inducing stimuli.

Some antiphlogistic drugs can influence IL-1 production. Paegelow and Werner (1989) reported that during calcium pyrophosphate-induced pleurisy in mice there was enhanced production of IL-1 in the pleural exudate and in serum.

Warner and Libby (1989) have shown that both human rTNF α and β induced transient accumulation of IL-1 mRNA by human vascular smooth muscle cells. TNF α treatment also increased intracellular biologically active IL-1 and subsequent release of IL-1 activity from the cells. Nawroth *et al* (1986) found that specific interaction of TNF with human umbilical vein endothelial cell receptors leads to the synthesis and release of IL-1.

IL-1 is also an autoregulator of IL-1 production. Manson *et al* (1989) found that IL-1 can stimulate its own production by human blood mononuclear cells and that this effect is IL-1 concentration dependent.

4.1.3. The biological properties of IL-1

Bronson *et al* (1987) found that the amount of hyaluronate in the medium of normal dermal fibroblasts was increased approximately 10-fold in the presence of IL-1. Postlethwaite *et al* (1988) observed that both human recombinant IL-1 α (rIL-1 α) and β stimulate the synthesis and accumulation of type I procollagen chains by fibroblasts. They also found that both types of IL-1 stimulate synthesis of tissue inhibitor of metalloproteinase, prostaglandin E₂, and growth of fibroblasts *in vitro*.

IL-1 induced a significant influx of PMN in a mouse subcutaneous sponge implantation model (Mason and Epps, 1989), in a rabbit inflammation model (Rampart *et al*, 1989) and in a mouse intraperitoneal injection model (Sayers *et al*, 1988). Martin and Resch (1988) showed evidence that IL-1 is chemotactic for mononuclear phagocytes and lymphocytes.

It has been demonstrated that the synthesis of plasminogen activator inhibitor (PAI) by cultured human and bovine endothelial cells was enhanced by IL-1 (Gramse *et al*, 1986; Nachman *et al*, 1986). Medina *et al* (1989) reported that treatment of bovine aortic endothelial cells with IL-1 dramatically increased the level of PAI messenger RNA. Bevilacqua *et al* (1985) reported that pretreatment of cultured human umbilical vein endothelial monolayers with IL-1 resulted in an increase in human peripheral blood PMN and monocyte adhesion.

Schmidt *et al* (1984) found that monocyte derived IL-1 was able to

regulate fibroblast proliferation, which suggests that the local release of IL-1 may contribute to abnormal connective tissue deposition in silicosis and other fibrotic diseases by stimulating fibroblast proliferation and amplifying other signals stimulating the synthesis of connective tissue components.

IL-1 also has a negative control of cell functions. Cavender and Edelbaum (1988) reported that both human rIL-1 α and β totally inhibited the increases in endothelial cell RNA synthesis, protein synthesis, and cell volumes induced by TNF and lymphotoxin. Cominelli *et al* (1990) demonstrated that pretreatment with IL-1 β before the induction of colitis reduces inflammation by a mechanism that requires prostaglandin synthesis.

IL-1 also exerts its role as an important mediator in inflammation and immunity via other interleukins. Sironi *et al* (1989) showed that *in vitro* exposure to recombinant IL-1 β markedly increased IL-6 production by human endothelial cells and IL-1-treated human endothelial cells expressed high levels of IL-6 mRNA. Dinarello (1989) suggested that IL-1 has effect on IL-2 production from T-cell lines. However IL-1 itself does not induce IL-2 secretion but requires a second signal, such as mitogen.

Presumably all biological effects of IL-1 are mediated by the high affinity binding of IL-1 to its receptor (Martin *et al*, 1988). A variety of cell types are known to have IL-1 receptors, such as cell lines of T lymphoma, epithelial cells, fibroblasts (Ohkawara *et al*, 1989) as well as human and murine PMN (Parker *et al*, 1988). However, little is known concerning the biochemical events following IL-1 receptor interactions. Oppenheim *et al* (1986) suggested that IL-1 does not induce intracellular changes in calcium concentration directly, but does depend on calcium channel formation for its effects.

4.1.4. IL-1 Inhibitor

Roberts *et al* (1986) reported that an IL-1 inhibitor was produced by human macrophages exposed to influenza virus or respiratory syncytial virus. Alveolar macrophages from normals and allergic asthmatic patients were also found (Gosset *et al*, 1988) to produce an IL-1 inhibitory factor.

Schwarz *et al* (1987) found that the fractions from ultraviolet (UV) irradiation-treated murine epidermal cells and from transformed keratinocytes eluting at around 40 kilodaltons inhibited IL-1 activity. In another study (Schwarz *et al*, 1988), they demonstrated the presence of a specific epidermal cell derived inhibitor of IL-1 in the sera of mice irradiated with UV.

Tiku *et al* (1986_b) found that normal human PMN are a source of a specific IL-1 inhibitor. The inhibitor is constitutively present in the PMN and

inhibits IL-1 in a dose-responsive manner. Arend *et al* (1989) described an IL-1 inhibitor produced by human monocytes cultured on adherent immune complexes or adherent IgG. The inhibitor specifically blocked binding of IL-1 to its receptor on the murine thymoma cell line EL4-6.1.

4.1.5. Production of IL-1 and pathogenesis

Fireman *et al*, (1989_a and _b) found that IL-1 secretion by alveolar macrophages was greatly increased in sarcoid patients but within the normal limits in patients with idiopathic pulmonary fibrosis. However, Yamaguchi *et al* (1989) found that the amount of IL-1 released from LPS-stimulated alveolar macrophages from smokers as well as pulmonary sarcoidosis was significantly decreased compared with that in nonsmokers. Eastgate *et al* (1988) demonstrated that the mean plasma IL-1 β was significantly higher in patients with rheumatoid arthritis than in healthy controls of similar age.

Becherucci *et al* (1989) found that rat macrophages collected either from exudates in pertussis-induced air pouches or from the peritoneum during adjuvant arthritis released more IL-1 when stimulated with LPS than cells from control rats. Lassalle *et al* (1990) demonstrated higher levels of spontaneous IL-1 secretion by alveolar macrophages from patients with coal worker's pneumoconiosis than by those from healthy controls.

Kusaka *et al* (1990_a) reported that both high rank and low rank coal mine dusts caused substantial release of IL-1 from normal alveolar macrophages *in vitro* and the levels were higher than those caused by quartz. In an *in vitro* study, alveolar macrophages from low rank coal mine dust- or titanium dioxide- exposed lungs showed increased ability to release IL-1 *in vitro*. They also showed (Kusaka *et al*, 1990_a) that quartz-elicited bronchoalveolar leukocytes, especially neutrophils, elaborated increased IL-1.

4.2. Tumour necrosis factor

Tumour necrosis factor (TNF) is produced primarily by activated macrophages. It acts via specific cell surface receptor and possesses an extensive array of biologic activities. Besides its *in vitro* cytotoxicity against various tumor cell lines, TNF also induces *in vivo* hemorrhagic necrosis in several tumors (Schollmeier, 1990).

4.2.1. Cells producing TNF

Macrophages are the major source of TNF and these cells can be stimulated substantially with LPS to increase secretion. TNF is produced by all types of macrophages thus far tested, including macrophages of pulmonary, hepatic, peritoneal, and bone marrow origin (Beutler and Cerami, 1988_a).

Cell maturation, as well as differentiation along mononuclear phagocytic line, is essential for TNF biosynthesis. Martinet *et al* (1988) found that LPS-activated alveolar macrophages expressed TNF transcripts, synthesized and released TNF whereas resting alveolar macrophages did not. Activated alveolar macrophages released several-fold more TNF than did autologous blood monocytes. Becker *et al* (1989) and Rich *et al* (1989) also demonstrated that maturation of mononuclear phagocytes *in vitro* enhanced their ability to produce TNF. They found that TNF production by LPS-stimulated alveolar macrophages peaked at approximately 8 h and ended after 24 h.

Kriegler *et al* (1988) found that activated monocytes synthesize a novel, rapidly inducible cell surface cytotoxic integral transmembrane form of TNF.

TNF is also produced by lymphocytes (Cuturi *et al*, 1987; Bendtzen, 1988) and endothelial cells (Dinarello, 1989). As reported by Beutler and Cerami (1988_a), T-lymphocytes produce abundant quantities of TNF mRNA and protein when induced with the calcium ionophore A23187 in conjunction with phorbol myristate acetate (PMA). Steffen *et al* (1989) showed the presence of TNF mRNA in the cytoplasm and TNF protein in the granules of individual human basophil/mast cells. Young *et al* (1987) have also demonstrated the presence of TNF responsible for natural cytotoxicity in murine mast cells.

4.2.2. Control of TNF biosynthesis

Controlled modulation of cell-derived protein mediators, such as TNF, is essential since these molecules not only orchestrate the inflammatory response, but also are crucial to normal cellular growth and differentiation (Kunkel *et al*, 1989).

Endotoxin enhances TNF mRNA transcription and induces TNF mRNA translation (Becker *et al*, 1989) as well as TNF secretion compared to monocytes without LPS stimulation (Nissen-Meyer *et al*, 1988). Collart *et al* (1986) reported that interferon (IFN) γ , by itself, is apparently not capable of stimulating release of macrophage-derived TNF, but it potently augments LPS-induced TNF release. Priming the monocytes with rIFN- γ before LPS exposure resulted in an increase in TNF production (Nissen-Meyer *et al*, 1988). The molecular mechanism of this enhancement occurs at both the transcriptional and post-transcriptional levels.

Besides endotoxin and IFN- γ , other compounds have been shown to be capable of inducing TNF production in monocytes/macrophages, e.g., protein kinase C-activating agents, immune complexes and the complement protein C_{5a} (Schollmeier, 1990).

Dexamethasone and other glucocorticoids can markedly suppress the production of TNF by LPS-treated macrophages when given a pre-treatment or

added along with the challenge (Beutler *et al*, 1986). The work of Kunkel *et al* (1988) indicated that prostaglandin E₂ (PGE₂) regulated macrophage-derived TNF gene expression and resulted in reduction of genomic transcripts for TNF. Kunkel *et al* (1989) suggested that PGE₂ acts as an endogenous autocoid with regulatory activity for TNF production. This regulation appears to be mediated via the intracellular accumulation of cAMP.

4.2.3. TNF production in pathological situations

Borm *et al* (1988) have investigated the release of TNF from blood monocytes in a group of coal miners and non-dust-exposed individuals. They found that monocytes from dust-exposed individuals revealed higher TNF release as compared to normal controls.

A rat study showed that after given by intratracheal instillation, silica caused increased release of TNF by alveolar macrophage than titanium dioxide did (Driscoll *et al*, 1990₁). Piguet *et al* (1990_a) showed that TNF plays an important part in silica-induced pulmonary fibrosis in mice since a single instillation of silica leads to a marked increase in the level of lung TNF mRNA and silica-induced collagen deposition is almost completely prevented by anti-TNF antibody, but is significantly increased by continuous infusion of mouse rTNF.

4.2.4. Inhibition of TNF

As it seems likely that TNF mediates several *in vivo* activities that can profoundly effect the well-being of an individual, it is clear that TNF activities must be diligently regulated. As with almost any soluble factor, TNF activities could be controlled at any of the several steps from gene transcription to the final step in the response of a target to TNF binding (Kunkel *et al*, 1989).

Evidence was presented by Lin *et al* (1988) that spleen cells from normal, untreated mice produce a factor that inhibits TNF cytolytic activity. Seckinger *et al* (1989) reported that urine of some febrile patients contains a TNF α inhibitory activity. Characterization indicated an apparent molecular mass of 33,000 Dalton. TNF α inhibitor blocks the different biologic activities induced by TNF α by interfering with the interaction of the ligand and its receptor (Lin *et al*, 1988; Seckinger *et al*, 1989). The further study of Seckinger *et al* (1990_a) indicated that the TNF α inhibiting activity originally described might be a soluble form of the TNF receptor itself. They purified the inhibitor and found that it forms a stable complex with TNF α and regulates TNF α bioactivity at the protein level (Seckinger *et al*, 1990_b).

4.2.5. The biological effect of TNF

Evidence that TNF plays a beneficial role by mediating resistance to a

wide range of organisms in a variety of infectious disorders is growing (Beutler and Cerami, 1988_a). However, inflammation also carries risks of its own. It is not difficult to understand that excessive production and release of TNF may rapidly lead to pathological changes (Beutler and Cerami, 1988_b).

Endothelium is an important target for TNF. Brett *et al* (1988) and Goldblum *et al* (1989) reported that TNF increases the permeability of endothelial cell monolayers to macromolecules and lower molecular weight solutes by a mechanism involving a pertussis toxin-sensitive regulatory G protein. However, the work of Gaskill (1988), using a rat model, suggested that TNF does not affect the permeability of the endothelial plasma membrane. Varani *et al* (1988) showed that pre-treatment of rat pulmonary artery endothelial cells with TNF increased their sensitivity to killing by PMN stimulated with PMA or C_{5a} in a time- and dose- dependent manner.

TNF was shown to modulate distinct PMN functions. Kapp *et al* (1989) demonstrated that TNF was a potent activator of the oxidative metabolism of human PMN. Ozaki *et al* (1988) found that TNF was a weak direct activator of oxygen radical production by PMN. However, pretreatment of PMN with TNF markedly potentiated oxygen radical production induced by opsonized zymosan. They suggested that the products of the lipoxygenase pathway play an important role in mediating the effects of TNF on PMN. Richter *et al* (1989) described that TNF could activate single adherent human PMN by causing the secretion of lactoferrin from secondary granules and myeloperoxidase from primary granules. Regulatory G- protein and local changes in the concentration of cytoplasmic Ca²⁺ were instrumental in this TNF-induced PMN degranulation.

Ozaki *et al* (1988) confirmed that TNF possessed chemotactic activity for PMN. Mason and Epps (1989) described, by using a mouse subcutaneous sponge implantation model, that injection of both TNF and C_{5a} stimulated an influx of PMN. In the study of Ming *et al* (1987) human rTNF was found to be chemotactic for both monocytes and PMN.

Palombella *et al* (1987) showed that TNF can increase the number of epidermal growth factor (EGF) receptors on human FS-4 fibroblasts. In another study, they reported that simultaneous addition of TNF and EGF to FS-4 fibroblasts grown in serum-free medium resulted in a synergistic stimulation of DNA synthesis and cell growth (Palombella *et al*, 1988).

Elias *et al* (1988_a) demonstrated in their study that TNF is important stimulator of fibroblast glycosaminoglycan biosynthesis. In another study, Elias (1988_b) showed that TNF could interact with IL-1 or IFN to inhibit the

proliferation of normal diploid fibroblasts via prostaglandin-dependent mechanisms since this inhibition was reversed when fibroblast prostaglandin production was blocked (Elias *et al*, 1988_a). Mauviel *et al* (1988) reported that TNF caused inhibition of collagen production by human dermal fibroblasts in a dose-dependent manner and the effect of TNF is exerted before the processing steps of procollagens.

Larrick *et al* (1987) demonstrated that rTNF caused activation of human granulocytes since granulocytes incubated with rTNF showed a dose-related increase in nitroblue tetrazolium dye reduction, in granulocyte polarization, in superoxide anion release, and in visually apparent aggregation. Pichyangkul *et al* (1988) found that after treatment with TNF α , the expression of surface adhesive proteins was increased on human granulocytes.

4.3. Comparison of IL-1 and TNF and synergism between them

4.3.1. Comparison of IL-1 and TNF

TNF and IL-1 are two distinct groups of proteins. Although direct comparison of the linear amino acid of TNF and IL-1 reveals no homology and there is also no overlap in receptor binding between the members of each group, these cytokines have many related and overlapping biological functions (Larrick and Kunkel, 1988; Dinarello, 1988).

The effects of IL-1 or TNF on a variety of cells *in vitro*, as well as systemic effects *in vivo*, are often biologically indistinguishable. Both IL-1 and TNF stimulate prostaglandin E₂ and platelet-activating factor production by cultured endothelium (Dinarello, 1989). Both of them stimulate procoagulant activity, leukocyte adherence, fibroblast proliferation and synthesis of plasminogen activator inhibitor (Dinarello *et al*, 1986). Both TNF and IL-1 induce the release of collagenase and arachidonate metabolites by cultured synovial cells and fibroblasts (Larrick and Kunkel, 1988). Both rIL-1 and rTNF induced dose-dependent PMN migration into rat peritoneal cavities (Faccioli *et al*, 1990) and release of a factor by macrophages into the supernatant *in vitro* which induced PMN migration. Both IL-1 and TNF induce IL-2-receptors, IL-6, colony stimulating factors and acute phase proteins (Oppenheim *et al*, 1989).

The most likely explanation of these related functions is that TNF and IL-1 stimulate similar intracellular messages and alter cellular metabolism in a similar way (Dinarello, 1988). Oppenheim *et al* (1989) believed that the overlapping effects of IL-1 and TNF are in part due to the induction of the same spectrum of cytokines and their receptors. IL-1 and TNF are also frequently released together by cells (Oppenheim *et al*, 1989).

The activity of TNF differs dramatically from that of IL-1 in that IL-1 is not toxic to a variety of tumor targets for which TNF is a potent cytotoxin (Dinarello, 1989). Rampart *et al* (1989) reported that IL-1 and TNF exert their pro-inflammatory effects *in vivo* via different mechanisms since, in an *in vivo* model of inflammation, IL-1-induced PMN leukocyte accumulation was slower in onset and was inhibitable by actinomycin D and cycloheximide. In contrast, TNF-induced PMN leukocyte accumulation was rapid in onset and very short of duration. Sayers *et al* (1988) reported that about 100 times more TNF than IL-1 protein was required to produce a comparable PMN infiltration in the peritoneal cavity.

4.3.2. Synergism between IL-1 and TNF.

When IL-1 and TNF are used together in experimental studies, the net effect often exceeds the additive effect of each cytokine (Dinarello, 1989).

IL-1 and TNF, when injected intradermally together, act synergistically in inducing PMN infiltration (Movat *et al*, 1987). Wankowicz *et al* (1988) and Sayers *et al* (1988) also found that there was synergy between TNF α and IL-1 in the induction of PMN migration during inflammation.

It was shown that rIL-1 and TNF act synergistically in the production of fibroblast prostaglandin (Elias *et al*, 1987). They demonstrated (Elias *et al*, 1988) that both IL-1 and TNF alone has a mild stimulatory effect on fibroblast proliferation. In contrast, a dose-dependent inhibition of fibroblast proliferation was noted when fibroblasts were simultaneously exposed to rIL-1 and TNF and this inhibition appeared to result from IL-1 and TNF synergistically stimulating fibroblast prostaglandin production. Furthermore, Elias *et al* (1990) described that combining rIL-1 and rTNF resulted in a synergistic increase in expression of IL-1 α activity by fibroblasts.

Dinarello (1989) suggested that the synergism between the two cytokines was due to second messenger molecules rather than up-regulation of cell receptors.

4.4. Plasminogen activator and plasminogen activator inhibitor

4.4.1. Plasminogen activator

Plasminogen is the inactive precursor of plasmin, the central enzyme responsible for fibrinolysis. Plasminogen activators (PA) are a group of specific neutral, serine proteases that proteolytically convert plasminogen to plasmin (Goldfarb, 1983). Plasmin, directly or indirectly, can promote degradation of all components of the extracellular matrix (Blasi *et al*, 1987).

PAs are serine proteases which fall into two immunologically and

biochemically distinct types: tissue-type (t-PA) and the urokinase-type (u-PA). T-PA appears to be the primary mediator of vascular fibrinolysis; it is synthesized and secreted by endothelial cells as well as tumor cells (Hart and Rehemtulla, 1988). Binding to fibrin is necessary for effective activation of plasminogen by t-PA. U-PA is synthesized and secreted by many cell types at different stages in their life cycle, such as macrophages (Chapman *et al*, 1984), epithelial cells (Gross *et al*, 1990) and endothelial cells (Levin and Loskutoff, 1982). U-PA plays a central role in regulating extracellular proteolysis in a variety of normal and pathological processes involving cellular functions, tissue destruction and cell migration (Blasi *et al*, 1987; Hart and Rehemtulla, 1988).

4.4.1.1. Production of PA and its regulation

Unkeless *et al* (1974) showed that cultured thioglycollate- stimulated peritoneal macrophages synthesize and continuously release high levels of PAs whereas unstimulated macrophages do not. However, Drapier *et al* (1979) found that thioglycollate- elicited mouse peritoneal macrophages secrete PA into the extracellular medium during *in vitro* cultivation only after contact with serum.

Chapman *et al* (1984) found that normal alveolar macrophages synthesize and express a membrane bound urokinase-type PA. Robinson (1988) reported that normal human alveolar macrophages produced PA in a time- and plasminogen concentration- related manner.

Levin *et al* (1982) reported that cultured bovine endothelial cells produced both urokinase and tissue-type PAs. Gross *et al* (1990) described large quantities of u-PA activity both in conditioned media and cell lysates from epithelial monolayers in culture. Stimulation of alveolar epithelial cell monolayers with LPS or TNF increased the levels of secreted PA.

Colony-stimulating factor was shown to enhance the production of PA by J774 cells, a mouse monocyte-like macrophage precursor cell line (Chen and Lin, 1984). Sitrin *et al* (1987) described that the acute lung injury induced in rabbit by an intravenous injection of PMA was characterized by extensive intra-alveolar fibrin formation and the substantial increase of the level of PA activity in bronchoalveolar lavage was found to contribute to the extent of fibrin deposition.

Chapman *et al* 1986 reported that human alveolar fluid from normal controls contained PA. In contrast, alveolar fluid from patients with sarcoidosis or idiopathic pulmonary fibrosis had low or absent PA activity. However, total cell-associated PA was not substantially different between patients and normal subjects.

Whitaker *et al* (1982_c) showed that pleural mesothelial cells have potent

fibrinolytic activity. Donaldson *et al* (1988_c) confirmed that mesothelial cells have considerable PA activity *in vitro*. This activity is depressed by damage to mesothelial cells, such as insult caused by mineral dusts (Donaldson *et al*, 1988_a).

4.4.1.2. Biological effects of PA

PAs play a general role in cellular migration, tissue remodelling and invasive processes of tumour cells (Goldfarb, 1983).

Liotta *et al* (1981) demonstrated that glycoprotein and collagenous components of basement membrane were poor substrates for PA and plasmin alone was not sufficient to completely degrade the whole basement membrane. Therefore, plasmin, generated through the action of PA, was considered to play a role only in the degradation of noncollagenous components of basement membrane.

Chapman *et al* (1984) have examined the role of macrophage-derived PA in the degradation of both insoluble fibrin and elastin matrices. The results indicated that cells degraded an insoluble fibrin matrix in the presence of plasminogen whether or not the macrophages contacted the fibrin. Live macrophages also degraded insoluble elastin only when in contact with the elastin. In another study (Chapman *et al*, 1988), they observed that human alveolar macrophages can degrade glycoprotein and elastin elements of extracellular matrices by an enzymatic process and plasminogen has critical importance in this process. Bignon *et al* (1985) suggested that plasmin, derived from plasminogen activator, may affect the proteoglycan "coat" around the more fibrous matrix collagens and elastin.

4.4.2. Plasminogen activator inhibitor

PA is regulated by several mechanisms such as specific interaction with fibrin, the abundant plasmin inhibitor α_2 -antiplasmin as well as PA inhibitors (PAI) (Sprengers and Kluft, 1987). However, PAIs play an important role in fine regulation of location and extent of plasminogen activation.

Two distinct protease inhibitors specific for PAs have been identified: PAI type-1 (PAI-1), is the major PAI in plasma and is derived primarily from endothelial cells, platelets and some neoplastic cell lines. This inhibitor is involved in the regulation of plasma t-PA activity (Blasi *et al*, 1987; Sprengers *et al*, 1987). The other, PAI type-2 (PAI-2), first purified from placenta extracts, is released by cultured monocytes-macrophages of different origin, U937 cells and PMN (Blasi *et al*, 1987). It plays a role in the regulation of extracellular PA activity involved in tissue remodelling (Hart and Rehemtulla, 1988). These two inhibitors are proteins of MW about 50,000, they differ in immunological reactivity

and in some physiological characteristics (Blasi *et al*, 1987). Protease nexin-1 is the third immunologically different PA inhibitor group. It is synthesized by a number of anchorage-dependent cells including fibroblasts and kidney epithelial cells. It limits activation of secreted u-PA (Sprengers and Kluft, 1987).

4.4.2.1. Production and regulation of PAI

Evidence was provided that cultured endothelial cells produced a rapid inhibitor of u-PA and t-PA (Philips *et al*, 1984). A further study by Levin (1986) showed that human endothelial cells release two forms of a PAI: an active form that readily binds to, and inhibits, PAs and an latent form that has no anti-activator activity but which can be activated by denaturation.

Mouse peritoneal macrophages release a fibrinolytic inhibitor after stimulation by endotoxin *in vitro* (Chapman and Stone, 1985_a). The inhibitor blocked urokinase directly and was very similar to placental PAI. In another report, Chapman and his colleague (1985_b) demonstrated that human alveolar macrophages released an endotoxin-inducible urokinase inhibitor. Wohlwend *et al* (1987) reported that human monocyte/ macrophages produce PA specific inhibitors that form covalent complexes with u-PA.

Klimetzek and Sorg (1979) described that peritoneal washout macrophages and casein or proteose peptone-elicited macrophages released fibrinolysis inhibitors. Macrophages in serum-free cultures continued to release inhibitors for at least 48 h. Dong *et al* (1988) demonstrated in an experimental pancreatic ascites tumour that inside the tumour cell, PA is the predominant activity, but in the extracellular fluid, the opposite is the case.

Sitrin *et al* (1986) demonstrated that the least dense rabbit alveolar macrophages released 6 times greater procoagulant activity than the activity displayed by denser cells. Markus *et al* (1980) showed that PA was significantly elevated in lung tumors compared to normal lung and that u-PA was the predominant PA in lung tumors.

Zeheb *et al* (1987) reported that incubation of rat hepatoma cells with the synthetic glucocorticoid dexamethasone rapidly inhibits t-PA activity by inducing a specific PAI-1. Kruithof *et al* (1986) described a PAI from the histiocytic lymphoma cell line U-937 which is immunologically related to a PA-inhibitor from human placenta and the primary role of the PAI is to regulate the activity of u-PA. Idell *et al* (1988) reported that in oleic acid-induced lung injury, the procoagulant activity of bronchoalveolar lavage fluids of sheep was markedly increased, whereas fibrinolytic activity was either depressed or undetectable.

Kopitar (1981) reported the presence of a urokinase inhibitor in peripheral

pig leukocyte cytoplasm. In another study, Kopitar *et al* (1985) showed that a PAI which inhibits primarily u-PA was isolated from the cytosol of human peripheral leukocytes.

4.4.2.2. Modulation of PAI activity by cytokines

Cytokines, such as IL-1 and TNF, have been confirmed to play important roles in modulation of PAI production and its activity.

Medina *et al* (1989) confirmed that treatment of bovine aortic endothelial cells with either IL-1 or human rTNF dramatically increased the level of PAI mRNA. Schleef *et al* (1988) also found that both rIL-1 β and rTNF α decreased human umbilical vein endothelial cells t-PA and increased PAI-1 in a dose- and time-dependent manner. Nachman *et al* (1986) showed the induction of PAI synthesis by IL-1 in human cultured endothelial cells.

Michel *et al* (1989) demonstrated that rIL-1 β induced a rapid and significant accumulation of both t-PA and u-PA mRNA and type 1 and 2 PAI mRNA in fetal lung fibroblasts. Rogister *et al* (1990) reported that cultured rat astroglial cells treated with IL-1 released enhanced PAI. In contrast, Campbell *et al* (1991) showed that human rIL-1 inhibits PAI-1 production by human articular cartilage and chondrocytes.

Scarpatti and Sadler (1989) found that in endothelial cells treated with TNF, PAI-2 was induced in part by inhibiting mRNA degradation. However, PAI-1 was not affected by TNF. Georg *et al* (1989) described that TNF α induced PAI-1 antigen in the human fibrosarcoma cell line HT-1080, and PAI-1 and u-PA antigens in the human carcinoma cell line T-CAR1, t-PA antigen was not affected or slightly decreased.

Medcalf *et al* (1988) showed that Human rTNF stimulates gene transcription of PAI-1 and PAI-2, and simultaneously suppresses constitutive gene expression of t-PA in human fibrosarcoma cells. They proposed that a TNF-mediated anti-fibrinolytic state may cooperate with the induction of procoagulant activity to stabilize the fibrin deposits commonly found in inflamed tissue.

4.5. Growth factors

Besides controls intrinsic to the cell, cellular replication is modulated by exogenous growth factors, a class of molecules that instructs cells to synthesize DNA and divide (Bitterman *et al*, 1982). In addition, polypeptide growth factors also play roles in differentiation, development, chemotaxis and activation of inflammatory cells, tissue repair, and disease (Deuel, 1987).

Growth factors are synthesized and secreted by both normal and

transformed cells. Abnormal secretion of growth factors probably results in diseases characterized by a proliferative cellular response or by fibrosis (Deuel, 1987).

There are many classes of growth factor (Kelley, 1990), but only two growth factors, i.e., epidermal and fibroblast growth factors are discussed here. As suggested by Gospodarowicz (1985), the tissue range sensitivity of fibroblast growth factor complements and sometimes overlaps with that of epidermal growth factor.

4.5.1. Epidermal growth factor

Epidermal growth factor (EGF) is mitogenic for a variety of mesenchymal cells and epithelial cells in culture. EGF markedly stimulates proliferation of those cells and leads to enhanced keratinization and squame production (Gospodarowicz, 1985). It also delays the ultimate senescence of the cells, thereby increasing their culture lifetime (Rheinwald and Green, 1977).

Sundell and colleagues (1980) demonstrated that infusion of murine EGF intravenously in fetal lambs caused epithelial hyperplasia of the conducting airways, resulting in morphologically more mature-appearing lungs than found in twin control animals. Gross *et al* (1986) showed that EGF enhances the rate of surfactant production in fetal rat lung slices and subsequently augments cellular proliferation.

Connell and Rheinwald (1983) reported that human mesothelial cells grew rapidly in culture when provided with EGF. They adopted a fibroblastic shape, forming parallel, multilayered arrays at saturation density and reducing synthesis and content of their four major keratins.

Fotev *et al* (1987) showed that wound exudates stimulated DNA synthesis in normal quiescent mesothelial cells with macrophages as the principal components of the exudate responsible for the activity. Cultured macrophage-conditioned media also induced an increase in mesothelial replication. Their further study (Fotev *et al*, 1987) confirmed the presence of a mesothelial mitogenic factor in the supernatant of wound exudates and macrophage cell cultures.

EGF binds to a specific cell surface receptor (Kelley, 1990). The EGF receptor is internalized when activated by EGF and is subsequently degraded. Recycling of the EGF receptor is insignificant, thus the interaction of EGF with its receptor provides a negative feedback loop that "down-regulates" the potential for additional signaling by EGF (Deuel, 1987).

4.5.2. Fibroblast growth factor

The biological effects of fibroblast growth factor (FGF) *in vitro* can be seen with respect to cell transformation, migration, proliferation, differentiation, and

senescence. It also stabilizes cell phenotypic expression and extends the culture life span (Gospodarowicz, 1985).

The effects of FGF include stimulation of cellular transport systems, polyribosome formation, protein synthesis, ribosomal and tRNA synthesis, and eventually DNA synthesis followed by cell division (Gospodarowicz, 1985).

Leibovich and Ross (1976) found a macrophage-dependent factor that stimulates the proliferation of fibroblasts *in vitro*. Bitterman *et al* (1982) demonstrated that human alveolar macrophages stimulated with particulates and immune complexes may modulate, in part, the replication rate of alveolar fibroblasts by releasing a growth factor within the alveolar microenvironment. In another study, Bitterman and colleagues (1983) found that alveolar macrophages lavaged from the patients with interstitial lung diseases secreted measurable growth factor activity. Macrophages from patients with sarcoidosis and more intense inflammation secreted higher levels of fibroblast mitogens. The study of Rom *et al* (1988) suggested that the alveolar macrophage-derived FGF is similar to insulin-like growth factor-1, recognized as a progression factor for fibroblasts.

Phan *et al* (1987) examined the release of macrophage-derived FGF activity by murine peritoneal macrophages with regard to its regulation by arachidonate metabolites, and found that products of cyclooxygenase and lipoxygenase pathways are potentially important both as exogenous and auto- or self-regulators of macrophage derived FGF release.

4.5.3. Cytokines as growth factors

IL-1 and TNF are also able to stimulate the growth of fibroblasts and other cells and may play a role as growth factors.

Dinarelo (1984) demonstrated that IL-1 produced by mononuclear cells stimulates fibroblast proliferation and collagen production. Bronson *et al* (1987) showed that the amount of hyaluronate in the medium fraction of normal dermal fibroblasts was increased dramatically in the presence of IL-1.

However, it is presently unclear whether this represents a true growth factor function of IL-1 or rather that IL-1 acts to increase the production of receptors for endogenous growth factors (Dinarelo, 1988).

Palombella *et al* (1987) reported that TNF can increase the number of EGF receptors on human FS-4 fibroblasts and this increase may be related to the mitogenic action of TNF in these cells. They suggested (Palombella *et al*, 1988) that EGF was responsible for driving the majority of the cell into S phase, while TNF appeared to make the cells more responsive to the mitogenic action of EGF.

However, some studies indicated that the synergy between IL-1 and TNF lead to the inhibition of fibroblast proliferation via prostaglandin-dependent mechanisms (Elias, 1988_a).

4.6. Asbestos and monokines

4.6.1. Asbestos and IL-1

Godelaine and Beaufay (1989) showed that the *in vitro* exposure of mouse peritoneal macrophages to chrysotile stimulated them to release IL-1. At equal doses, quartz was a more potent inducer than chrysotile. However, injury caused by chrysotile or quartz to macrophages did not merely cause a leakage of IL-1 from the cells, since the intracellular levels of the mediator were increased rather than decreased upon exposure to these particles.

The immunostimulatory effects of respirable mineral dusts on Wistar-rat alveolar macrophages and T-lymphocytes were studied *in vitro* (Oghiso and Kubota, 1987). Silica and crocidolite, but not titanium-dioxide, were found to significantly stimulate production of IL-1 by the macrophages.

Kagan *et al* (1985_a) showed that inhalation of either crocidolite or chrysotile asbestos in the rat is associated with augmented release of immunostimulatory substances having both IL-1 and IL-2 functional activities. Kagan *et al* (1989) found that these asbestos-related effects were most pronounced in the higher density-defined (younger) macrophage fractions. The effects of crocidolite exposure tend to be more prominent and to persist for a longer period after the cessation of asbestos inhalation exposure than chrysotile. Since IL-1 activity is controlled by the I-region of the major histocompatibility complex, the effect of asbestos inhalation on alveolar macrophage Ia antigen expression was also investigated (Kagan *et al*, 1985_a). Asbestos inhalation is associated with enhanced Ia antigen expression on alveolar macrophages, an effect which is more pronounced after crocidolite than after chrysotile exposure.

Rosenthal *et al* (1989) showed that there was no IL-1 production by alveolar macrophages *in vitro* in the absence of LPS stimulation in control or fibre exposed cultures. However, after addition of suboptimal concentrations of LPS, chrysotile and crocidolite asbestos markedly stimulated IL-1 production.

In the study by Hartmann *et al* (1984), rats were exposed, by intermittent inhalation, to crocidolite, chrysotile asbestos or clean air and then were immunized with fetal calf serum antigens. Macrophage-lymphocyte co-culture supernatants, when obtained from immunized, asbestos exposed rats, contained greater IL-1 and IL-2 activity than identical supernatants from immunized, sham exposed animals. These differences between group were not, however, observed in supernatants

from unimmunized rats, or when supernatants were generated in the absence of immune lymphocytes. These observations suggest that asbestos exposure is associated with enhancement of immune responses.

4.6.2. Asbestos and TNF

Dubois *et al* (1989) demonstrated that chrysotile asbestos and silica augmented TNF production by alveolar macrophages in a concentration-dependent manner in culture. Moreover, the use of the TNF α -specific antisera indicated that TNF represents most of fibroblast growth activity generated by mineral dust-stimulated alveolar macrophages. They also found that endogenous leukotrienes, such as leukotriene B₄, produced by alveolar macrophages play a role in the cellular events which lead to enhanced TNF activity in response to asbestos and silica particles.

Rosenthal *et al* (1989) reported that in the absence of LPS stimulation, TNF production by alveolar macrophages was minimal in control or fibre exposed cultures. When incubated with suboptimal concentrations of LPS, alveolar macrophages exposed to chrysotile and crocidolite asbestos secreted high TNF activity.

4.6.3. Asbestos and PA and PAI

Hamilton *et al* (1976) described that intraperitoneal injection of asbestos into mice induces the formation of exudates containing macrophages that produce PA. *In vitro*, the addition of asbestos to macrophage cultures also stimulated PA secretion. The *in vitro* treatment of mouse peritoneal macrophages by asbestos stimulated the expression of plasma membrane bound PA activity (Lison *et al*, 1989).

Cantin *et al* (1989) described that macrophage and BAL fluid PA activity was significantly increased in asbestos exposed sheep with abnormal X-rays, whereas no such change was seen in exposed sheep with normal X-rays. Workers with no signs of asbestosis had normal BAL fluid PA activity whereas PA activity was sharply increased in those with early signs of asbestosis. However, PA activity in workers with confirmed asbestosis was within the normal range.

Godelaine *et al* (1989) found that both normal and LPS-activated mouse peritoneal macrophages induced increasing fibrinolysis after exposure to UICC chrysotile asbestos, but there was a longer lag period for normal macrophages. Strong stimulation of PA release by chrysotile or crocidolite-treated macrophages was observed compared to slight stimulation by rockwool and glass fibres. Donaldson *et al* (1988_c) showed that PA activity of rat mesothelial cells was inhibited after treatment with non-toxic doses of crocidolite asbestos.

4.6.4. Asbestos and growth factor

Lemaire *et al* (1985_c) demonstrated that supernatants from bronchoalveolar lavage cells of asbestos intratracheally exposed rats stimulated tritiated thymidine incorporation by lung fibroblasts to a greater extent than supernatants from control rats. In another study, Lemaire *et al* (1986_a) reported that alveolar macrophages from asbestos intratracheally exposed rats showed a significantly increased production of FGF and the increase persisted up to 6 months. Concanavalin-A stimulated blood mononuclear leukocytes from asbestos-treated rats showed reduced fibroblast growth inhibitory activity. Lemaire *et al* (1986_a) suggested that local production of FGF may be a contributing factor in the development of asbestos-induced pulmonary fibrosis, whereas systemic suppression of fibroblast growth inhibitory activity may represent a consequence or an amplifying factor of the fibrotic process.

Lemaire *et al* (1986_b) also evaluated the effects of supernatants of BAL cells on lung fibroblast proliferation and found that supernatants of BAL cells from rats which had received intratracheal instillation of chrysotile asbestos had significantly higher FGF activity than control levels.

Adamson and Bowden (1990) demonstrated that BAL macrophages from rats intratracheally exposed to short fibre asbestos secreted factors that enhanced both proliferation and secretion of collagen by fibroblasts in culture, but there was no evidence of a fibrotic response in these animals. Conversely, exposure to long fibre crocidolite caused no increase in the secretion of FGFs by BAL macrophages, but did induce pulmonary fibrosis. The authors contended that FGF release by interstitial macrophages might be far more important in inducing pulmonary fibrosis than the release of growth factor by BAL macrophages. The ability of fibres to cross the epithelium may thus be an important descriptor of their pathogenicity.

Wiedemann *et al* (1985) showed that amosite asbestos fibres enhanced the production of a mesothelial cell-derived soluble factor with molecular weight greater than 12,000 daltons which stimulates fibroblast DNA synthesis. Renier *et al* (1990) reported that rat pleural mesothelial cells treated with chrysotile or crocidolite showed a significant dose-dependent increase in [³H]dThd incorporation compared to untreated cells and further study indicated that asbestos produces unscheduled DNA synthesis.

PURPOSE AND SCOPE OF THE STUDY

The foregoing literature review has shown that, despite a substantial body of research into the role of leukocytes in fibre-related lung disease, the pleura has attracted limited attention. A number of studies, with emphasis on pleural pathological change resulting from asbestos inhalation or pleural inoculation, however, have been done. Few publications have focused on the characterization of the pleural leukocytes.

In the present study, intratracheal asbestos instillation in PVG and HAN rat models were used throughout. The aims of the study were:

- (1). to determine whether or not intratracheal injected-asbestos could reach the pleural cavity.
- (2). to investigate the changes in pleural leukocyte populations and in their cytokine-producing abilities as well as fibrinolytic activity induced by intratracheal asbestos.
- (3). to compare activation of pleural leukocytes from intratracheal *C.parvum*-exposed rat lung with those from asbestos-exposed lung.
- (4). to compare the functions of alveolar leukocytes and pleural leukocytes after intratracheal instillation of asbestos.
- (5). to investigate the interaction of pleural leukocytes and pleural mesothelial cells and influence of asbestos on the interaction.
- (6). to assay the effects of asbestos in culture on alveolar macrophages cytokine production.

It was hoped that the study would yield information of general relevance to the mechanism of pleural injury arising from asbestos-exposure.

MATERIALS AND METHODS

1. Animals

Syngenic, PVG rats from the Institute of Occupational Medicine's Animal Unit were used throughout. Male or female rats, 12 weeks of age or greater at the commencement of experiments were used. WISTAR-derived rats of the HAN strain, purchased from the Western General Hospital Animal Centre, Edinburgh, both male and female and 12 weeks of age or more, were also used where indicated.

2. Asbestos and other mineral dusts

The Union Internationale Contre Le Cancer (UICC) standard asbestos samples of crocidolite, amosite and chrysotile (A) were used in the study. Specially prepared long and short amosite fibres were also used (Donaldson *et al*, 1989_a). The long fibre amosite was generated as a cloud in an exposure chamber and the airborne fibres were found to have a size distribution substantially longer than that of the standard UICC amosite. The long fibre sample used in the study was collected from the chamber air on to filters. Preparation of the short fibre amosite samples was as follows: a quantity of the bulk long fibre sample described above was ground in a ceramic ball mill and sedimented in water, comprehensive analysis of the final sample showed no loss of crystallinity and an elemental composition close to the parent long amosite sample (Donaldson *et al*, 1989_a). Fibre length and diameter distributions of the long and short fibre amosite dust clouds were described earlier by Davis *et al* (1986_a) in which fibres $> 0.4 \mu\text{m}$ in length and with an aspect ratio $> 3:1$ were sized by scanning electron microscopy at a magnification of $\times 10,000$. Almost all fibres were less than $5 \mu\text{m}$ in length for short fibre amosite and long fibres were prepared from raw amosite that contained a high proportion of long fibres greater than $20 \mu\text{m}$.

The titanium dioxide (TiO_2) was the rutile form supplied by Tioxide Limited (Stockton-on-Tees). The quartz used was the DQ₁₂ standard sample (Messrs, Dorentuper Sand-und Thonwerke GmbH of Dorentup, Westphalia) (Robock, 1973).

3. Use of *C.parvum* to study the result of inflammation in the alveolar and pleural spaces

Heat-killed *Corynebacterium parvum* was obtained from Wellcome Reagents, Beckenham. When required for intratracheal instillation, a volume of 0.2 ml, containing 1.4 mg of *C.parvum*, was injected via the trachea into the lungs.

4. Exposure of animals and cells to asbestos fibres and other dusts

4.1. Intratracheal instillation of dusts

Rats were anesthetized with ether, the trachea exposed and a small

incision was made and a blunt-ended needle introduced. A volume of 0.5 ml of Dulbecco's phosphate-buffered saline (PBS), containing required doses of asbestos, dusts or 0.2 ml of *C. parvum* suspension, was then injected, and the skin closed with metal clips. Control rats were uninjected or injected with PBS alone. It was found that there was no difference between these two controls in our studies. The injected rats were killed at intervals up to one month and lavages were then carried out.

4.2. Dust exposure *in vitro*

Bronchoalveolar lavage leukocytes were suspended in F10 medium (Gibco, Paisley) containing 2% bovine serum albumin (BSA) (Sigma, Poole) at a concentration of 1×10^6 /ml. A suspension of the test dust in PBS was added into the culture at the desired concentration and then cultured for 24 hours.

4.3. Long term inhalation of asbestos and/or quartz for the transmission electron microscope study

Dust clouds were generated in 1 m^3 exposure chambers. The asbestos dust clouds were produced using a modified Timbrell generator. The quartz dust cloud was generated using a turntable generator to obtain better control at the low dose levels required. The planned exposure levels were 10 mg/m^3 of respirable asbestos and 2 mg/m^3 of quartz. Since in the laboratory, routine monitoring of dust clouds is by mass, using an MRE sampler, asbestos or particulate dust was generated separately for part of each day at a concentration calculated to produce the required mean over the whole seven hour exposure period. The asbestos clouds were generated for five hours each day, while the particulate dusts were generated for two hours each day (Davis *et al*, 1986_a).

Rats of the HAN strain were treated by combined exposure to asbestos and particulate dusts for one year. Most of the animals were allowed to live out their full life span except that some rats were killed at the end of the dusting period and six months or even shorter time period later. Bronchoalveolar and pleural lavage to obtain leukocytes were carried out.

5. Lavage techniques

5.1. Bronchoalveolar lavage

Rats were killed by intraperitoneal injection of Nembutal (Ceva Ltd, Watford) and the trachea cannulated with a blunt, 18- gauge needle secured with nylon thread. The lungs were removed from the thorax and bronchoalveolar lavage was performed by injecting 8 ml of PBS at 37°C into the lungs via the cannula. PBS was then withdrawn after massage and the entire procedure was repeated 4 times.



5.2. Pleural lavage

To obtain pleural leukocytes, pleural lavage was carried out by inserting a round-ended 8-gauge cannula through the parietal pleura between the lower two ribs and injecting 5 ml PBS at 37 °C into the pleural space monitored via the opened peritoneal cavity. The PBS was then withdrawn after massaging the rib cage and this procedure was carried out 4 times.

Pleural lavage fluid was obtained by injecting 2 ml F10 medium plus 2% BSA into the pleural cavity, then withdrawing after massaging prior to pleural lavage. The fluid was spun at 1,000 rpm for 10 min, the cells discarded, and the fluid retained.

6. Preparation of cell supernatants

Cell suspensions were spun at 1,000 rpm for 5 min and resuspended in F10 medium with 2% BSA to obtain a concentration of 1×10^6 /ml and incubated at 37 °C, 5% CO₂ for 24 hrs. For leukocyte stimulation, lipopolysaccharide (LPS, Sigma, Poole) was added into the culture at 100 ng/ml. The supernatants were collected and centrifuged at 3,000 rpm for 10 min to remove cell debris, then stored at -70°C prior to assay.

7. Separation of pleural leukocytes

Pleural leukocyte suspension at 6×10^6 /ml in F10 medium + 2% BSA were thoroughly mixed with Sepracell (Sepracell-MN, Sepratech Corporation, Oklahoma) at a ratio of 1:3. The mixture was then spun at 3,000 rpm for 15 min. The fraction at the top of Sepracell was macrophage enriched and the fraction on the bottom was the mast cell-enriched population. The fractions were transferred into universals and washed 3 times with F10 medium, and resuspended in F10 medium plus 2% BSA.

8. Preparation of Cell lysate

Whole or separated pleural leukocyte populations, at 1×10^6 /ml, were frozen in liquid nitrogen and thawed in 37 °C water bath immediately. This procedure was repeated three times and the suspensions were then spun at 3,000 rpm for 10 min to get rid of cell debris. This soluble fraction from the lysed cells will be henceforth referred to as the lysate.

9. Primary culture of rat mesothelial cells

Mesothelial cells which were isolated from scrapes of the peritoneal cavity and frozen down at early passage (Donaldson *et al*, 1988_a) were recovered from liquid nitrogen. The cells were then cultured in F12 medium (Flow Laboratories, Rickmansworth) containing 10% fetal calf serum (FCS, Gibco, Paisley) until required, when cells were spun down and resuspended in F12 medium plus 2%

BSA.

10. Fibrinolysis assay

10.1. Measurement of plasminogen activator (PA) activity

The plasminogen-dependent and -independent fibrinolytic activity of bronchoalveolar leukocytes and mesothelial cells were measured using iodinated fibrinogen according to the modified chloramine T method of Unkeless *et al* (1974). A pH 7.5, 0.25 M buffer was prepared as follows: 2.5 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ (May & Baker Ltd, Dagenham) was dissolved in 400 ml distilled water and 14.25 g Na_2HPO_4 (anhydrous, May & Baker Ltd, Dagenham) in 50 ml distilled water using heat to aid solution; these were then mixed and made up to 500 ml with distilled water. For the labelling of fibrinogen with $\text{Na}^{125}\text{-Iodine}$ (Na^{125}I), 10 μl of a 1 mg/ml solution of fibrinogen (Sigma, Poole) was made up in the buffer solution, which had been filtered through a 0.2 μm membrane (FlowPore D, Flow laboratories, Sartorius, West Germany) was placed in the bottom of a conical tube and then 5 μl of Na^{125}I (containing 500 μCi) (Amersham International, Amersham) was added. Then, 50 mg chloramine T (Sigma, Poole) was dissolved in 20 ml of 0.25 M buffer and 10 μl of the solution was added to the tube and mixed gently for 15 secs. L-cysteine hydrachloride (Sigma, Poole) 10 mg was then dissolved in 10 ml of 1:5 diluted buffer and 100 μl of this solution was added to the tube and mixed. Finally, 50 mg KI (Sigma, Poole) was dissolved in 10 ml buffer and 1 ml of this solution was added to the tube, then mixed gently. Free ^{125}I was removed from the above mixture by chromatography on a Sephadex G25 column (PD-10 column, Pharmacia, Milton Keynes) equilibrated with PBS containing 0.01% fibrinogen. The ^{125}I -fibrinogen was eluted with 0.01% fibrinogen solution and up to 0.5ml x 25 fractions were collected.

The efficiency of iodination was examined by trichloroacetic acid (TCA, BDH Chemical Ltd, Poole) precipitation. Five μl aliquot of each fraction plus 50 μl of FCS were mixed with 2 ml of 20% TCA and the radioactivity was counted. The sample was then spun at 2,500 rpm for 10 min, the supernatant poured off and the TCA precipitated counts were measured. The bound [^{125}I]-fibrinogen normally accounted for more than 95% of the total counts in the labelled fractions (Fig. M.1). The fractions containing the radiolabelled fibrinogen (first peak) were combined and stored in refrigerator prior to use.

^{125}I -labelled fibrinogen was diluted in PBS with 1 mg/ml unlabelled fibrinogen so that an 80 μl aliquot contained 1×10^5 cpm. Aliquots of 80 μl of this solution were add to wells of Falcon microtiter plates (Linbro, Flow Laboratories, Rickmansworth) and the plates were dried at 45°C for 3 days. The

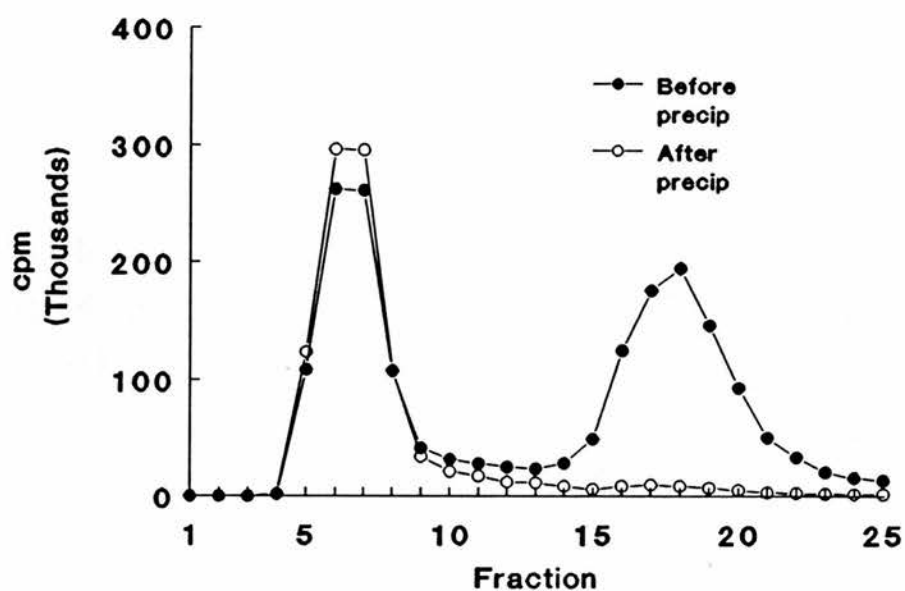


Figure. M.1 Effect of TCA precipitation on the radioactivity (cpm) of $[^{125}\text{I}]$ -fibrinogen

Each point represents the mean of 2 separate experiments performed in triplicate. SD, which were not presented for clarity, varied from 5-75% of means. The first peak is fibrinogen-bound ^{125}I and the second peak is free ^{125}I .

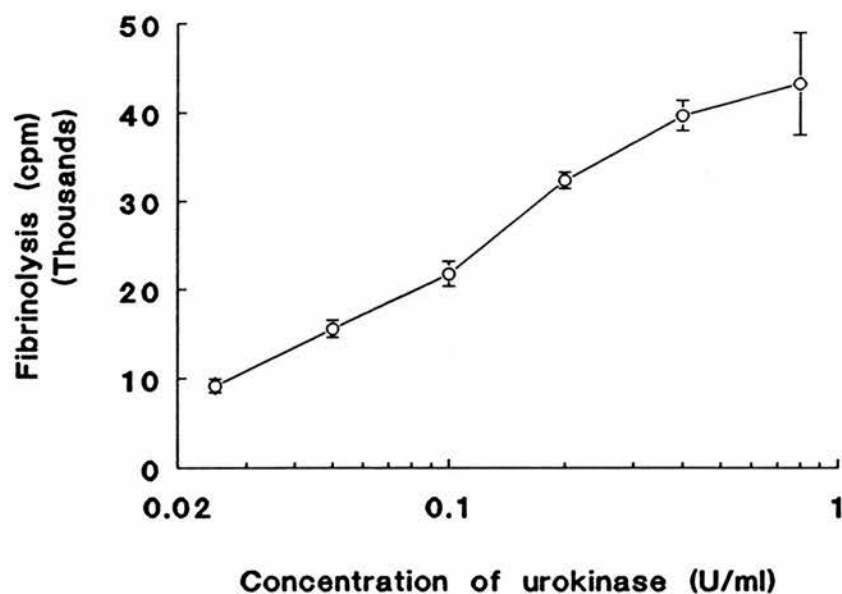


Figure M.2 Effect of concentration of urokinase on Fibrinolysis

Results are presented as mean and SD of one experiment in triplicate. The concentration of PLG was $1 \mu\text{g/ml}$.

wells were then incubated at 37 °C for 4 hr with 100 μ l F10 medium containing 2.5% freshly thawed FCS to convert fibrinogen to fibrin. The plates were washed twice with 200 μ l PBS prior to use.

The cells were incubated either with or without 1.2 μ g/well plasminogen (PLG, Kabi, Stockholm and a gift from Dr. I.McGregor, Blood Transfusion Centre, Edinburgh) in 200 μ l F10 plus 2% BSA for bronchoalveolar cells and in 200 μ l F12 plus 2% BSA for mesothelial cells, to measure PA activity. The plates were incubated at 37 °C in a 5% CO₂/95% air mixture for 24 hr and 150 μ l of aliquot was sampled from each well to assess the solubilized [¹²⁵I]fibrin. The assay was carried out in triplicate.

10.2. Measurement of plasminogen activator inhibitor (PAI)

To determine whether pleural leukocytes secrete PA inhibitor, pleural leukocytes or their supernatant were added to cultures of bronchoalveolar leukocytes and mesothelial cells (both at 0.25 x 10⁶/ml) with or without PLG for 24 hr, to assess their effects on fibrinolysis. The dose-responses of pleural leukocytes and their supernatants were determined. The background counts were normally about 400 cpm both with and without PLG in the assay.

10.3. Characterization of PAI in pleural leukocyte supernatant

The fibrinolysis assay was used for simultaneously measuring both PA and PAI activities in cell supernatants or lysates. This was achieved by adding urokinase and PLG to [¹²⁵I]fibrin plates to a concentration that causes a moderate degree of fibrinolysis and then observing if the addition of supernatant or lysate enhanced or decreased fibrinolysis.

The concentration of urokinase (kindly provided by Dr. I.McGregor, Blood Transfusion Centre, Edinburgh), plasmin (Sigma, Poole) and streptokinase (Sigma, Poole) were used at a concentration causing 50% of total [¹²⁵I]fibrinolysis. The activity of PAI in the pleural leukocyte cells and supernatants was determined by titration against urokinase and PLG. In our assay, the concentrations of PLG and urokinase were chosen at 1 μ g/ml (excessive dose) and 0.05 U/ml, respectively (Fig. M.2). Solubilized [¹²⁵I]fibrin was assessed as described above. For typical urokinase and PLG-mediated fibrinolysis, the counts of spontaneous release were about 4,000 cpm. The counts of trypsin-treated wells gave approximately 25,000 cpm.

The PAI activity was expressed as percent inhibition of PLG-dependent fibrinolysis caused by urokinase in the presence of diluted supernatants, compared with the fibrinolysis in the absence of supernatants. PAI activity was determined according to the following equation:

$$\text{Percent inhibition} = \frac{(\text{Fib. without Sup.} - \text{blank}) - (\text{Fib. with Sup.} - \text{blank}) \times 100}{(\text{Fib. without Sup.} - \text{blank})}$$

where units of fibrinolysis = cpm, Fib. = fibrinolysis, Sup. = supernatant.

To partially characterize the PAI, the activity of the inhibitor in the pleural leukocyte supernatant after incubation at 50°C or 70°C for 1 hr was compared with that of an untreated supernatant. To assess the molecular weight of the inhibitor, the supernatant was centrifuged using a centricon-10 microconcentrator (Amicon Division, W.R. Grace & Co., Danvers), with a 10,000 D cut off.

For further characterisation of PAI in pleural leukocyte supernatant, the acid-resistance of PAI in pleural leukocyte supernatant was determined. The supernatant was first treated with 2N HCl at PH 3.5 for 2 hrs and then adjusted to PH 7.4 with 2M NaOH. The supernatants were sterilised by filtering through 0.2 µm membrane. The supernatants before or after acid-treatment were measured for PAI activity in the above assay.

11. C3H mouse thymocyte proliferation assay for detecting IL-1 activity

IL-1 activity in supernatants was quantified using the C3H mouse thymocyte assay (Mizel, 1981). Mice, age at 2-4 week, were killed by ether overdose (May & Baker Ltd, Dagenham, England). Thymus glands were removed and single cell suspensions of thymocytes were prepared by disaggregation. The cells were washed and suspended at $6 \times 10^6/\text{ml}$ in RPMI-1640 medium (Gibco, Paisley) with 10% FCS, 20 µM 2-mecaptoethanol (Sigma, Poole) and 25 mM hepes buffer (BDH Chemical Ltd, Poole), PH 7.1-7.4 adjusted with 1M NaOH. One hundred µl of the cell suspension were added to each well, together with 50 µl of RPMI medium containing 20 µg/ml of phytohemagglutinin (PHA, Sigma, Poole) and 50 µl of dilutions of control RPMI medium or test pleural leukocyte supernatants. The plates were then incubated at 37°C in 5% CO₂ for 48 hr. The cultures were pulsed with 0.25 µCi ³H-methyl-thymidine per well (Amersham International, Amersham). After one more day of culture, the cells were harvested onto glass filters, and ³H incorporation was quantitated by scintillation spectrophotometry. The activity of test samples was calculated by comparing with the standard curve (Fig. M.3) of a series of dilutions of IL-1β standard (A gift from Dr. D. Burnett, Lung Immunobiochemical Research Laboratory, General Hospital, Birminham).

12. L929 cell-lytic assay for measuring TNF activity

Murine L929 cells (from Dr. Julian Symonds, Department of Rheumatology,

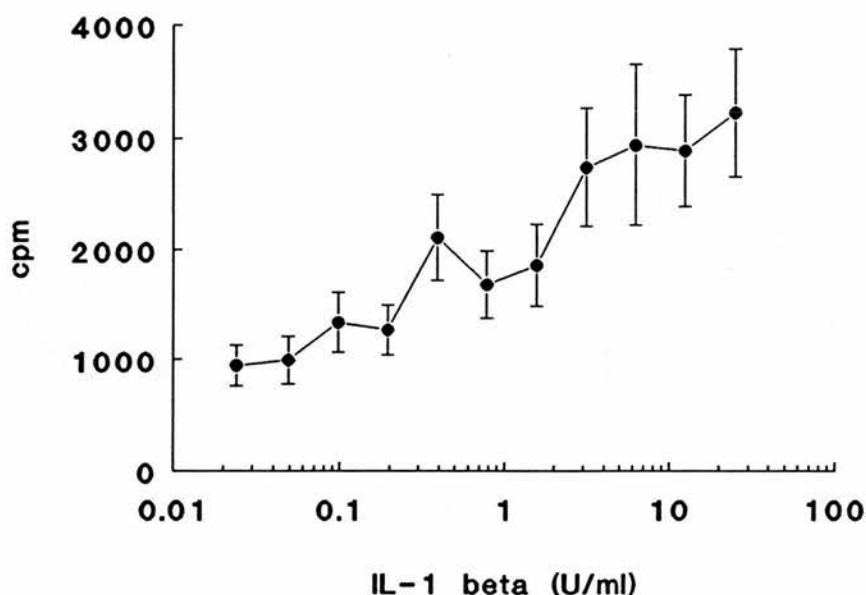


Figure M.3 IL-1 β standard curve

Standard curve was produced by plotting concentration of IL- 1 β standard against thymocyte proliferation as measured by [^3H] thymidine uptake. Each point represents the mean and SD of 12 separate experiments in triplicate. The equation of standard curve was $Y = 2086 + 374 \ln X$, Y: cpm; X: IL-1 β activity, U/ml; $r=0.92$.

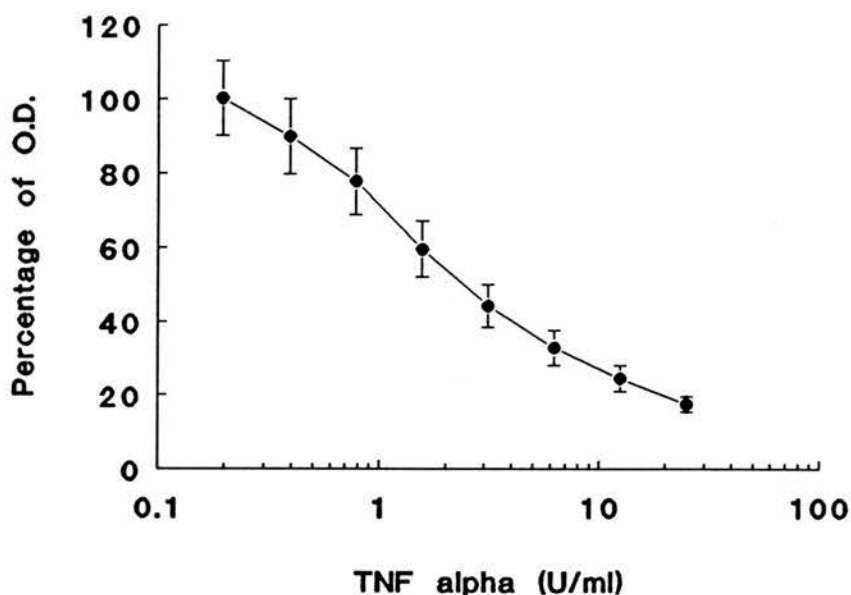


Figure M.4 TNF α standard curve

Standard curve was obtained by plotting TNF concentration against activity in L929 survival as measured by O.D. Each point stands for the mean and SD of 8 separate experiments in triplicate. The equation of the curve was $Y = 65.68 - 14.14 \ln X$, Y: percentage of sample O.D. to control O.D., X: TNF α activity, U/ml; $r=0.93$.

Northern General Hospital, Edinburgh) were grown in minimal essential medium (MEM medium, Gibco, Paisley) containing 5% FCS. For use in the TNF assay (modified according to Warner et al, 1989), confluent, monolayers were trypsinized and suspended in assay medium (MEM + 5% FCS, no antibiotics) at a concentration $0.3 \times 10^6/\text{ml}$. One hundred μl aliquots of cell suspension were added to each well of a 96-well microtiter plate and the plates incubated at 37°C , 5% CO_2 for 24 hr. The supernatant was then aspirated and discarded. One hundred μl of assay medium containing 1 $\mu\text{g}/\text{ml}$ actinomycin D (Sigma, Poole) were added to all wells. Fifty μl of pleural leukocyte supernatants and 50 μl of assay medium with 2 $\mu\text{g}/\text{ml}$ of actinomycin D were added to experimental wells in triplicate; these were then double-diluted using a multichannel pipette. Following 18–20 hr incubation, the plates were emptied by inversion then stained with 100 $\mu\text{l}/\text{well}$ of crystal violet methanol solution. The plates were washed with water and allowed to dry. Optical density at 540 nm was determined with a MR650 plate reader (Dynatech Laboratories Inc, USA). TNF activity in the supernatant was determined by comparison with a $\text{TNF}\alpha$ standard (a gift kindly supplied by Dr. Julian Symonds, Department of Rheumatology, Northern General Hospital, Edinburgh) dilution curve (Fig. M.4).

For confirming the TNF activity in alveolar and pleural leukocyte supernatants, a rabbit anti-TNF serum (kindly supplied by Dr. Joseph Fantone and Dr. Steven Kunkel, Ann Arbor, Michigan, USA) was used in our assay. Pre-bleed rabbit serum was used as a control. Fifty μl of suitable concentration of pleural leukocyte supernatant or TNF standard with 2 μg actinomycin D and 50 μl of serial dilutions of rabbit TNF anti serum or control serum were added into microtiter plate with L929 cells, then incubated and stained as above.

13. Use of CTLL-2 cell proliferation assay to measure IL-2 activity in supernatants of pleural leukocytes

The IL-2 dependent cell line CTLL-2 cells were cultured in RPMI medium with 10% FCS supplemented with 5% IL-2 enriched rat splenocyte supernatant in the absence of hepes. When required, the cells were washed twice with fresh medium and resuspended in the medium at concentration of $1 \times 10^5/\text{ml}$. Fifty μl of cell suspension were dispensed into each well of microtiter plates, followed by the addition of 50 μl of test supernatants. Plates were incubated for 24 hours at 37°C in a 5% CO_2 and 95% air atmosphere. Each well was labelled with 10 μl of ^3H -thymidine (0.25 μCi) and then incubated for one further day. ^3H -thymidine uptake by CTLL-2 cell was measured to determine IL-2 activity in supernatant.

14. Endotoxin measurement and removal

Contaminating levels of endotoxin in the media used in the studies were determined by using a kit (COATEST, KABI Diagnostica, KabiVitram Ltd, Uxbridge, Middx).

Polymyxin column (Detoxi-Gel affinitypak columns, Pierce Chemical Company, Illinois, USA) were used to remove endotoxin from medium. An endotoxin depleting efficiency of 99% with these columns was found.

15. Macrophage and PMN chemotaxis assay

The chemotactic activity of pleural leukocyte supernatants was measured using Blindwell chambers (Donaldson *et al*, 1990). The target cells, macrophages were obtained by bronchoalveolar lavage of control rats (>95% pure) and PMN were obtained from rats one day after intratracheal instillation of 1.4 mg *C.parvum* (80% neutrophils). In the chemotaxis assay, two controls were used: the positive control was zymosan- (Sigma, Poole) activated rat serum, ie. rat serum which was incubated with 1 mg/ml of zymosan at 37 °C for 1 hr and then centrifuged at 3,000 rpm to remove the zymosan; the negative control was F10 medium plus 2% BSA alone. Two hundred µl of controls or test supernatants were placed in the lower compartment of the Blindwell chamber. To detect the chemotactic activity of supernatants for macrophages, a 5-µm nucleopore filter (Pleasanton, California) was placed on top. The top part of the chamber was screwed down and 400 µl of F10 medium + 2% BSA containing 6×10^5 macrophages were placed in the upper compartment. The chamber was then incubated for three and half hours at 37 °C in a moist 5% CO₂ atmosphere. The cell suspension in the top compartment was then pipetted gently up and down and decanted. The top compartment of the chamber was unscrewed and the filter carefully removed from the chamber, washed twice in PBS and stained with Diff Quick (Merz-Dade, Dudingen, Switzerland). The filters were allowed to dry before mounting in DPX plastic mountant under a cover slip. The macrophages which had migrated through the filter were counted at x1000 magnification by light microscopy. To measure the neutrophil chemotactic activity of pleural leukocyte supernatants, 3-µm in pore diameter filter (Pleasanton, California) was used and the incubation time was 45 min. Other procedures were the same as those for measurement of macrophage chemotactic activity.

16. Mesothelial cell lysis and detachment assay for determining mesothelial cell damage

Mesothelial cells were grown and serially passaged in F12 medium with 10% FCS until required. In the lysis and detachment assay (Donaldson *et al*,

1988_b), the mesothelial cells were plated into 96-well microtiter plate at 5×10^4 cells per well in 100 μ l F12 medium plus 10% FCS. The cells were labelled overnight with ^{51}Cr (Amersham International, Amersham) at 74 KBq per well in 100 μ l F12 medium + 10% FCS. In preliminary experiments, it was found that after 24 hr incubation, the spontaneous release of ^{51}Cr by mesothelial cells during 8 hr after incubation was less than 25% of total ^{51}Cr uptake as determined by lysis with 1% Triton-X-100 (Sigma, Poole) (Tab. M.1). After incubation, supernatants were removed and the monolayers were washed 2 times with 200 μ l of PBS. The lavaged pleural leukocytes were resuspended with F12 medium containing 2% BSA at different concentrations and 200 μ l of cell suspension were pipetted onto mesothelial cell monolayers to achieve final ratios between pleural leukocytes (effectors) and mesothelial cell (targets) of 0:1, 0.1:1, 1:1, 10:1 and 20:1. After co-culture of targets and effectors for 4 hr at 37°C, the microtiter plates were centrifuged for 5 min at 1,000 rpm. Release of soluble ^{51}Cr , a measure of cell lysis, was determined by aspirating 50 μ l of supernatant from each well, then transferring to tubes and counting in a γ counter. Cell lysis was expressed as cpm after being multiplied by four.

Table M.1 MESOTHELIAL CELL ^{51}Cr -CHROMIUM UPTAKE AND SPONTANEOUS RELEASE

Total Uptake	Time course of spontaneous release (hours)				
	0	2	4	6	8
14906(8372) ^a	1613(519)	1862(519)	2739(1056)	3169(1259)	3292(975)
	(10.8%)	(12.5%)	(18.4%)	(21.3%)	(22.1%)

^an=2 separate experiments in triplicate, results are presented as mean (SD) cpm.

The detachment of mesothelial cells was measured by aspirating the remainder of the supernatant of mesothelial cells, the monolayers of the cells were then washed with 200 μ l of PBS. This was repeated twice and combined washes were counted for ^{51}Cr activity of detached and loosely adherent cells. Final detached ^{51}Cr activity was determined by following equation: $Y = (X \times 3)^*$.

* Y: ^{51}Cr activity in remaining supernatants of mesothelial cells and two washes, X: ^{51}Cr activity in 50 μ l supernatant of mesothelial cells for measuring cell lysis.

17. Attempts to detect mesothelial cell growth factor in pleural lavage fluid

Mesothelial cells growing in flasks were trypsinized, washed and resuspended in F12 medium + 2% FCS at a concentration $0.25 \times 10^6/\text{ml}$; 200 μl of the cell suspension was then transferred into microtiter plate and incubated at 37°C , 5% CO_2 overnight. The medium was replaced by 50 μl of F10 medium + 2% BSA and 50 μl of diluted test supernatants or pleural lavage fluid. F10 + 2% BSA alone was used as the negative control. Meanwhile, each well was labelled with 0.5 μCi [^3H]-methyl-thymidine and further overnight incubation was carried out. The culture supernatants were then poured off and 200 μl of trypsin (Sigma, Poole) was added into wells. The cells were harvested on glass fibre filters after 10 min incubation at room temperature. The filters were dried and the cellular [^3H] thymidine incorporation was measured by liquid scintillometry in a β counter.

18. Observation of latex microspheres and *C.parvum* organisms in both bronchoalveolar and pleural lavage leukocytes

To study the possible transfer of particles from the alveolar to the pleural space, animals were intratracheally instilled with 0.4 ml of PBS containing a 1:20 dilution of monodispersed fluorescent carboxylated microspheres which have a geometric diameter of 0.770 μm (beads, Polysciences Inc., Warrington). The beads were injected together with *C.parvum* as required.

Cytospin preparation of bronchoalveolar and pleural leukocytes were observed by epi-fluorescent light microscopy to detect the beads. The macrophages and neutrophils from both populations were divided into two groups: -with or without beads. In the group with beads, the cells were further divided into 3 subgroups: -cells containing less than 5 beads, cells containing 6-10 beads and cells containing more than 10 beads; a differential count was also carried out.

In addition, following *C.parvum* exposure alone, the lavaged leukocytes were examined for intracellular organisms. Both macrophages and neutrophils were divided into two groups:-with or without intracellular *C.parvum* organisms.

19. Transmission electron microscope study of pleural structure and pleural leukocytes after instillation of asbestos

Fresh pleural lavage leukocytes were spun down and then suspended in 2.5% glutaraldehyde (BDH Ltd, Essex) in 0.1M sodium cacodylate (Fisons Scientific Apparatus, Loughborough, Leics) solution. The cell suspension was kept on ice for 2 hr and then washed three times with 0.1M sodium cacodylate solution. The leukocytes were resuspended in 2% osmium tetroxide (OsO_4 , BDH Ltd, Essex) in 0.1M sodium cacodylate solution for 2 hr and washed 3 times with

0.1M sodium cacodylate solution again. After the third wash, the supernatant was replaced by 1 ml 2% warmed liquid agar and spun at 1,000 rpm for 5 min. Following setting of the agar the solid pellet was removed from the tube and cut into 1 mm size cubes which were then stored in 0.1M sodium cacodylate solution prior to further processing (modified according to Robinson, 1977).

The sodium cacodylate solution was removed and 10% acetone (May & Baker Ltd, Dagenham) in distilled water was added and rotated on Rotary Mixer for 15 min, then decanted. This was replaced with 25%, 50% and 70% acetone solution sequentially. A 90% acetone solution was then added and rotated for 15 min, this was repeated twice followed by 3 changes of absolute acetone. Equal parts of absolute acetone solution and 100% epoxy resin (Araldite CY 212, Agar Scientific Ltd, Stansted, Essex) was substituted in and rotated for 3 hr. Finally, cell cubes were removed into 100% Araldite and rotated overnight. The cell cubes were then embedded into moulds filled with araldite, one cube to each mould, and polymerised at 60°C for 48 hr (Robinson, 1977).

For pleural structure studies, tissues at 1 mm³ size were cut from the visceral and parietal pleura of rats killed by intraperitoneal Nembutal injection. Fixation was the same as above except for the agar treatment stage.

The cell or tissue araldite blocks were squared off and then trimmed to form a "pyramid". When necessary, 1 µm resin sections were cut to examine and enable areas of interest in the block face to be pinpointed for subsequent ultramicrotomy. Sections were stained by covering them with a few drops of 1% methylene blue (BDH Ltd, Essex) and then heating the slide on a hotplate at 60°C - 70°C for a few minutes. Excess stain was washed off in running hot water and the slides were air-dried and examined under light microscope (Robinson, 1977).

Ultrathin sections were cut using glass knives made by a knifemaker (Ultracut; LKB Instrument Ltd, South Croydon, Surrey). Sections were collected on copper grids (Agar Aids, Stansted, Essex) which were then held on grid sticks (TAAB Laboratories Equipment Ltd, Reading). Sections were stained in a saturated solution of uranyl acetate (BDH Ltd, Essex) in 70% alcohol for 20 min and washed 3 times with 70% alcohol, then dried. They were then stained in lead citrate (BDH Ltd, Essex) solution (0.2% in 0.1M NaOH) for 5 min prior to rinsing in 0.1M NaOH solution. Finally, the sections were washed three times with distilled water and dried (Robinson, 1977).

The sections were examined using a CORA electron microscopy (CORA electron microscopy Ltd, Manchester) and Hitachi H-7000 electron microscope

(Hitachi Ltd, Japan).

20. Opsonization of amosite asbestos and mineral dusts

Rat whole blood was prepared and left at 37°C for 1 hr to allow clotting, then spun at 3,000 for 15 min. The serum was heated at 56°C, 1 hr for complement inactivation or non-treated. One mg/ml of dusts (long, short fibre amosite, DQ₁₂ or TiO₂) were incubated in PBS, heat-inactivated or non-inactivated serum, 50 µg/ml rat IgG (Sigma, Poole) or human fibronectin (Sigma, Poole) solution at 37°C for 30 min on a rotater. After incubation, the dust particles were washed twice with PBS and spun at 3,000 rpm, 10 min. After that, dust PBS-suspension was added to culture of control alveolar macrophages (1 x 10⁶/ml) at a concentration of 50 µg/ml and incubated overnight and TNF activity in the cell supernatants was determined.

For observing the effect of different doses of IgG on ability of dusts to stimulate TNF production by alveolar macrophages, 10, 100 to 1000 µg/ml of IgG were used to opsonize dusts. Further investigation was carried out to determine the possible enhanced effect of complement activation in opsonization with IgG. After opsonization with IgG the fibre were treated with heat-inactivated or non-heat-inactivated rat serum at 37°C for 30 min, then washed with PBS and resuspended in PBS.

21. Presentation of results and statistical analysis

Results were expressed as the mean ± standard deviation (SD) or mean ± standard error of mean (SEM) (when indicated in results) as an index of variation. Results were subjected to analysis of variance. When there was a significant F value for the effect of treatment, individual means were compared for significance using t test (Ryan *et al*, 1985). Significant difference of different groups was also analysed using Student's t test (Bailey, 1959).

RESULTS

1. Components of lavage leukocytes of normal rats

1.1. Normal PVG rat bronchoalveolar and pleural leukocyte populations

Table 1.1 shows the populations of the different leukocyte types present in lavages of the bronchoalveolar and pleural spaces. The alveolar leukocytes were mostly macrophages, whereas the pleural leukocytes contained substantial proportions of mast cells and eosinophils.

Table 1.1 DIFFERENT CELL TYPES PRESENT IN NORMAL PVG RAT BRONCHOALVEOLAR AND PLEURAL LEUKOCYTE POPULATIONS

	Total number	Differential percentage			
	(x10 ⁶)	Macrophages	Lymphocytes	Mastcells	Eosinophils
Bronchoalveolar					
leukocytes	4.8(1.5) ^a	97(3.2)	3(3.2)	0	0
Pleural					
leukocytes	4.5(1.0) ^b	82(5.6)	1(1.2)	11(5.4)	6(2.8)

^a_n=3 rats, ^b_n = 14 rats. Each result is presented as mean(SD). No neutrophils were seen in either normal cell populations.

Figures 1.1 to 1.6 show light microscope and transmission electron microscope pictures of components of pleural leukocytes.

1.2. Normal HAN rat bronchoalveolar and pleural leukocyte populations

The bronchoalveolar leukocyte populations of HAN rats were very similar to those of PVG rats (Table 1.2). The total number and components of pleural leukocytes of HAN rats, however, were slightly different to those of PVG rats. There were more lavagable pleural leukocytes from HAN rat pleural space than in PVG rat pleural space. Macrophages in the HAN rats accounted for a lesser percentage in whole pleural population whereas mast cells and eosinophils were proportionally greater compared with PVG rats.

Only when indicated, the results were obtained from experiments using HAN rats. Otherwise, the results were derived from PVG rats.

2. Pleural leukocyte responses to intratracheal instillation of crocidolite asbestos

2.1. The effect of intratracheal instillation of crocidolite asbestos on the pleural leukocyte populations

Figure 2.1 shows the differential pleural leukocyte count from rats at different times following intratracheal instillation (I/T) of 5 mg crocidolite asbestos. One day after I/T asbestos, the pleural leukocyte population was unaffected,

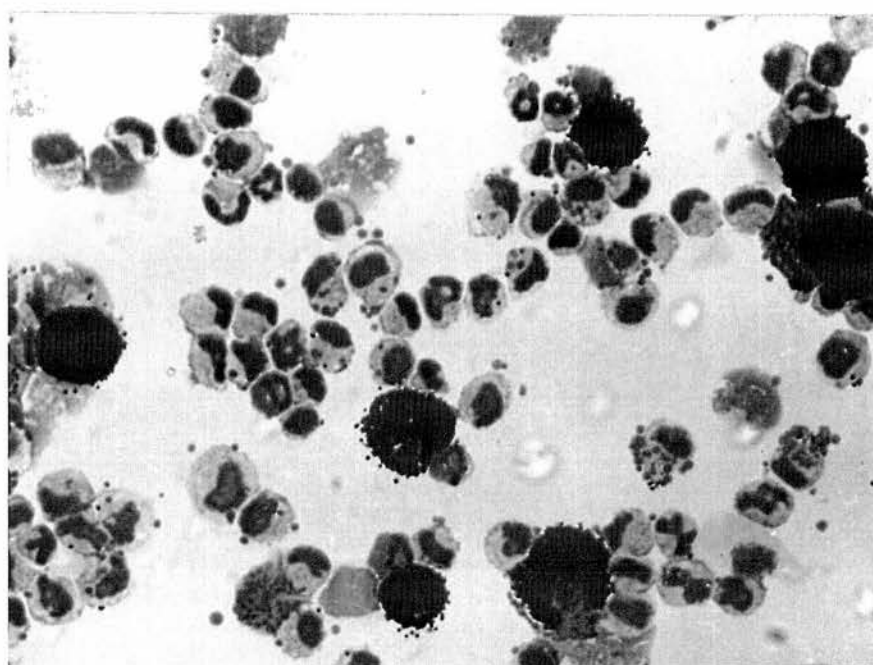


Figure 1.1 Components of normal pleural leukocytes (x200)

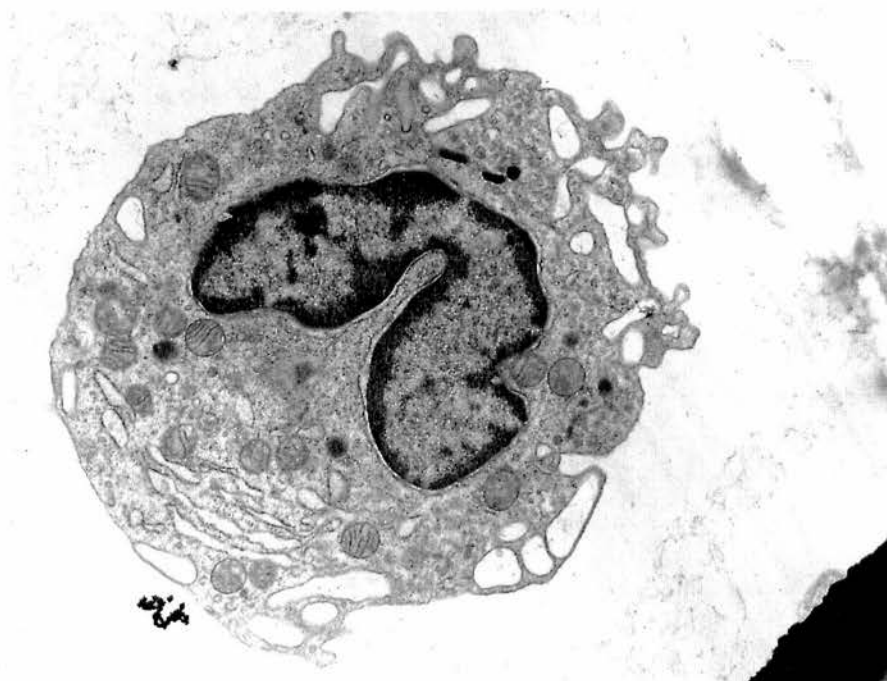


Figure 1.2 Transmission electron micrograph of normal pleural macrophage (x5,000)

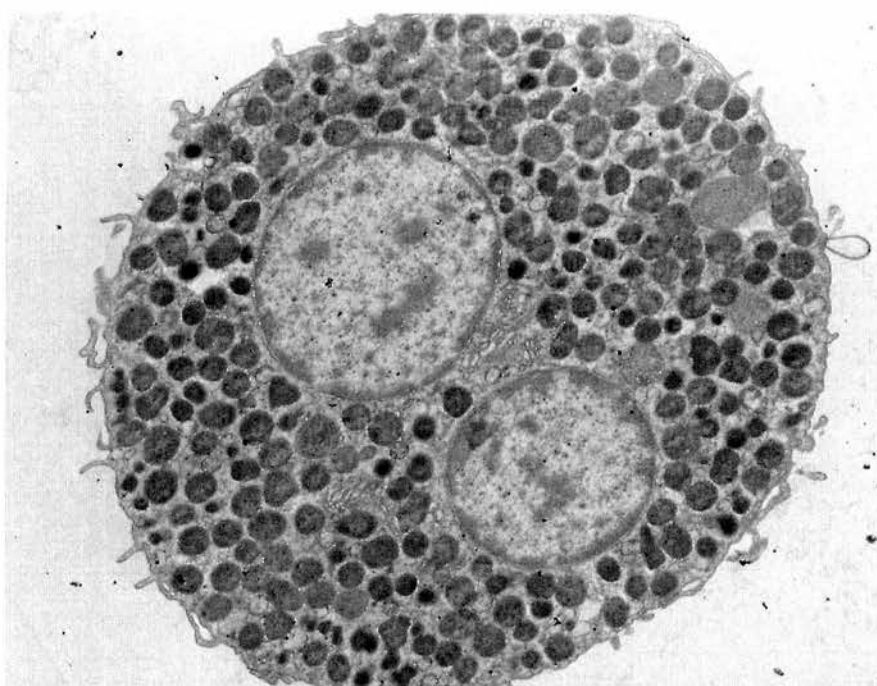


Figure 1.3 Transmission electron micrograph of normal pleural mast cell (x5,000)

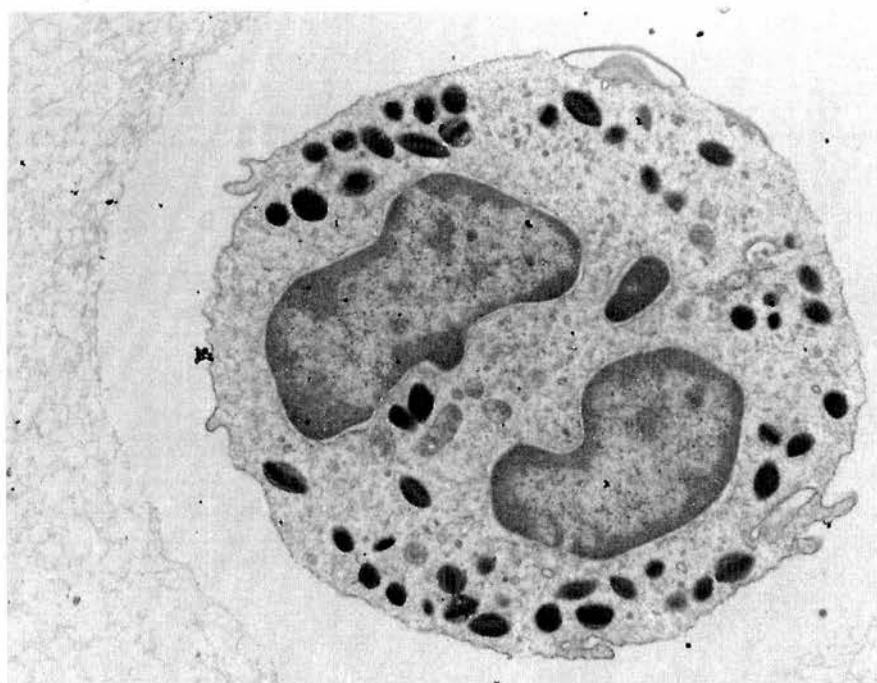


Figure 1.4 Transmission electron micrograph of normal pleural eosinophil (x5,000)

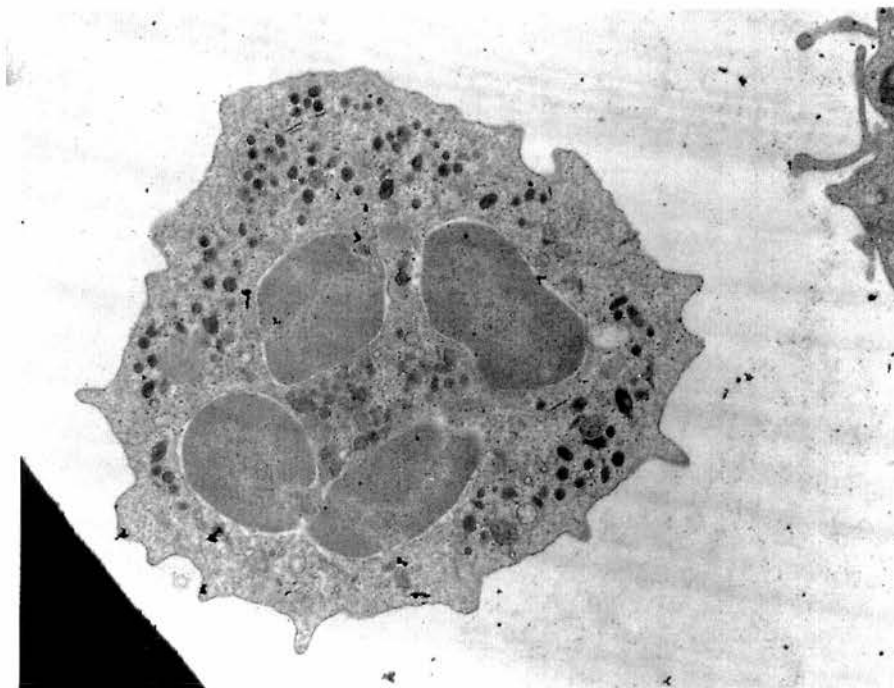


Figure 1.5 Transmission electron micrograph of pleural polymorphonuclear leukocyte after inhalation exposure to amosite and quartz (x5,000)

Polymorphonuclear leukocytes (PMN) were not normal resident in the pleural space. They appeared only during the pleural inflammation induced by intratracheal instillation of *C.parvum* or dusts, including asbestos, into the airspaces or inhalation of asbestos.

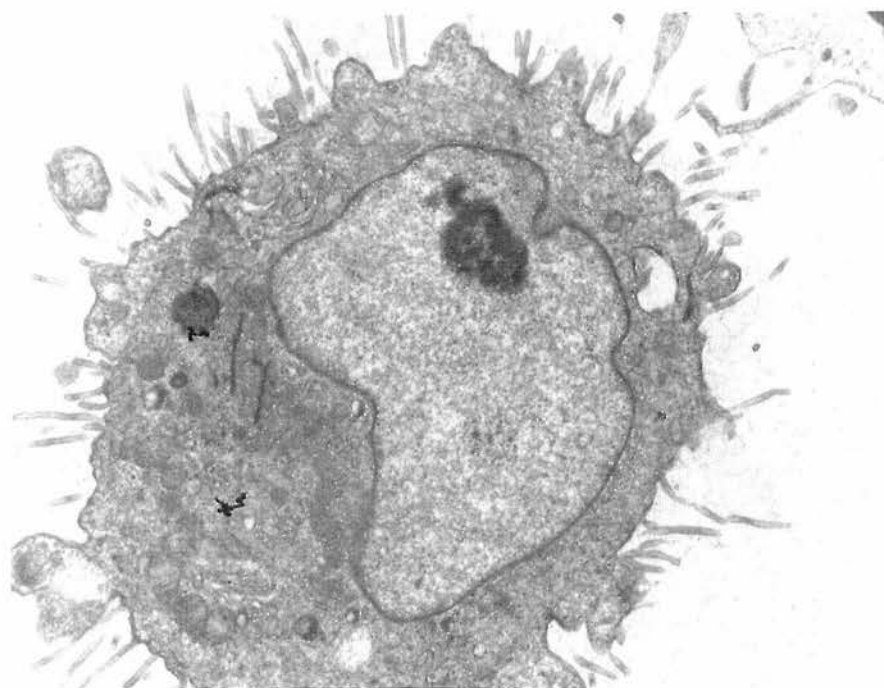


Figure 1.6 Transmission electron micrograph of pleural mesothelial cell (x5,000)

Pleural mesothelial cells were not normal components of the free cells in pleural space. However, following intratracheal instillation of asbestos or long term inhalation of asbestos, these cells were found occasionally in the pleural leukocyte population.

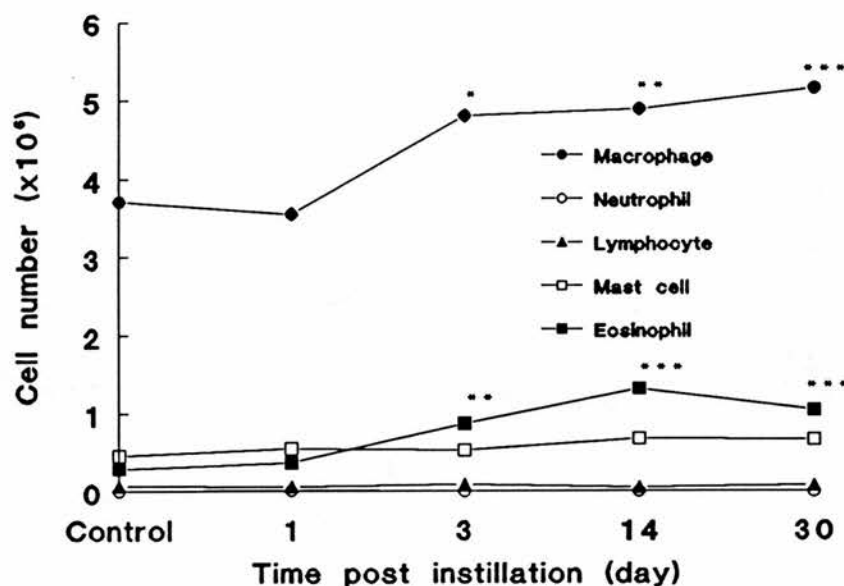


Figure 2.1 Differential numbers of pleural leukocytes from rats at various times after I/T 5 mg crocidolite asbestos

For clarity the standard error of means (SEM), which was less than 15% of the mean, is not presented in the figure. Each point represents mean of 5-13 separate experiments. Asterisk denotes a significant difference from control: $*=P<0.05$, $**=P<0.01$ and $***=P<0.001$.

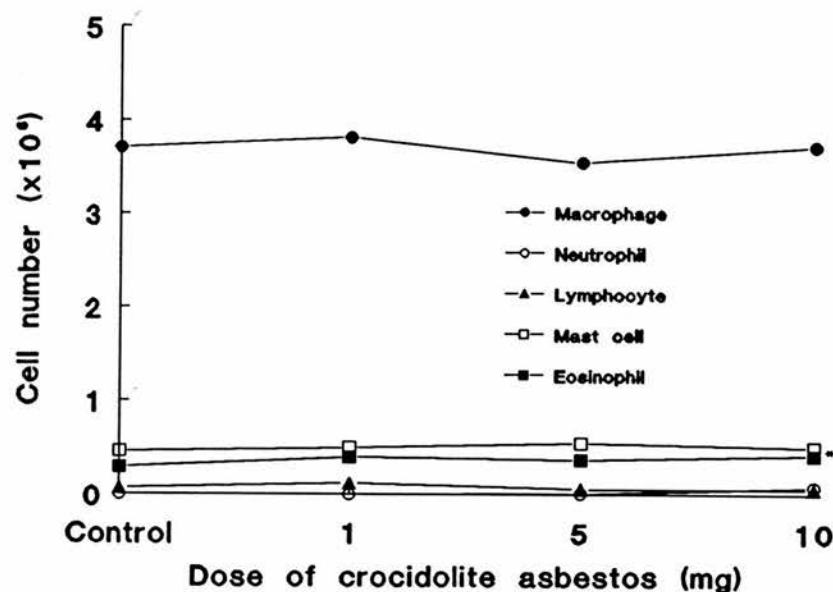


Figure 2.2 Differential numbers of pleural leukocytes from rats one day after I/T various doses of crocidolite asbestos

SEM, which was less than 15% of the mean, is not presented in the figure for clarity. Each point represents the mean of 5-13 separate experiments. Asterisk denotes a significant difference from control: $*=P<0.05$.

**Table 1.2 DIFFERENT CELL TYPES PRESENT IN NORMAL HAN RAT
BRONCHOALVEOLAR AND PLEURAL LEUKOCYTE POPULATIONS**

	Total number	Differential percentage			
	(x10 ⁶)	Macrophages	Lymphocytes	Mastcells	Eosinophils
<hr/>					
Bronchoalveolar					
leukocytes	5.0(2.7) ^a	99(0.7)	1(0.8)	0	0
Pleural					
leukocytes	8.7(3.0) ^b	71(2.6)	2(1.6)	15(2.4)	12(4.6)

^a_n=7 rats, ^b_n = 4 rats. Each result is presented as mean(SD). No neutrophils were seen in either normal cell populations.

From 3 days up to one month after 5 mg asbestos instillation, the total numbers of macrophages increased significantly compared with control pleural leukocytes. Meanwhile, eosinophils increased markedly, although other pleural leukocytes remained at the same level.

Figure 2.2 illustrates the populations of pleural leukocytes one day after instillation of various doses of crocidolite asbestos up to 10 mg. The higher doses of crocidolite caused a slight recruitment of neutrophils. However, other types of cells remained the same as controls.

2.2. Increased production of plasminogen activator inhibitor by pleural leukocytes after rats were instilled intratracheally with crocidolite asbestos

2.2.1. The production of plasminogen activator (PA) by normal alveolar macrophages and pleural mesothelial cells

Figure 2.3 shows that normal alveolar leukocytes induced plasminogen (PLG)-dependent fibrinolysis which had a dose-response to the concentration of alveolar leukocytes, there was no PLG-independent fibrinolysis. However, pleural leukocytes caused neither PLG-dependent nor PLG-independent fibrinolysis. The results indicated that alveolar leukocytes produced a plasminogen activator in culture. Figure 2.4 demonstrates that mesothelial cells induced PLG-dependent fibrinolysis, but not PLG-independent fibrinolysis.

2.2.2. The production of plasminogen activator inhibitor (PAI) by normal pleural leukocytes

To assay for the presence of the inhibitor from pleural leukocytes, both

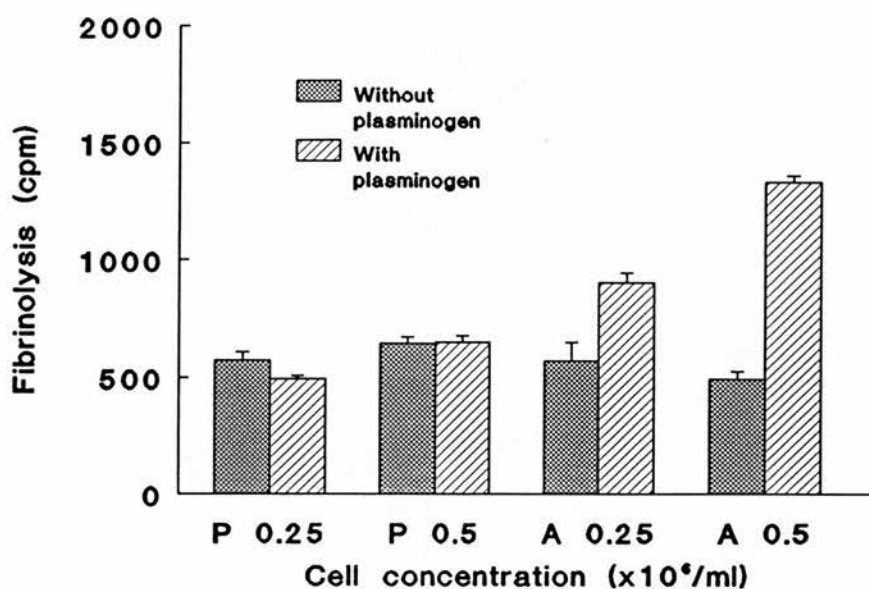


Figure 2.3 PLG-dependent and -independent fibrinolysis caused by alveolar and pleural leukocytes

Each bar represents the mean and SEM of triplicate wells in 3 separate experiments. P and A represent alveolar and pleural leukocytes respectively.

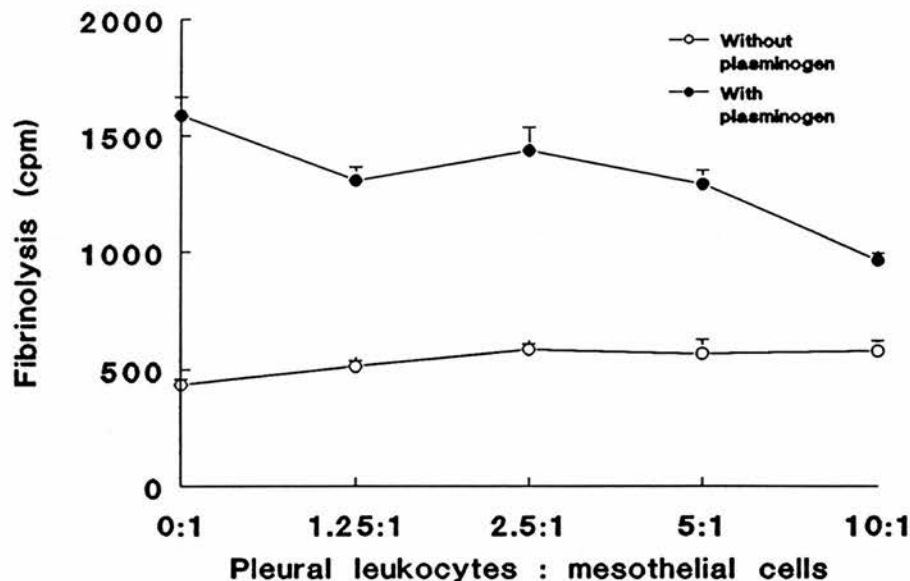


Figure 2.4 Effect of increasing concentrations of pleural leukocytes on mesothelial cell-induced fibrinolysis

Each point represents mean (SEM) of triplicate wells in two separate experiments.

alveolar leukocyte and mesothelial cell fibrinolysis were tested after the addition of pleural leukocytes or their supernatants. The fibrinolytic activity of both cells was inhibited by pleural leukocytes (Figs. 2.4, 2.5) and that of alveolar leukocytes was inhibited by pleural leukocyte supernatants (Fig.2.6). This reduction in fibrinolytic activity was dose-dependent with both pleural leukocyte number and supernatant dilution. In contrast, PLG-independent fibrinolysis by both populations remained at a very low level that was near the background and this was not inhibited by pleural leukocytes or their supernatants.

For characterization of the specific point of action of the fibrinolysis inhibitor, i.e., activity on plasmin or plasminogen activator, three different fibrinolytic systems were used: urokinase plus PLG, streptokinase plus plasminogen, and plasmin alone. The concentrations of urokinase, plasmin, and streptokinase were chosen to degrade about 50% of the [125 I]fibrin. Figure 2.7 illustrates the effect of pleural leukocytes and their supernatants on fibrinolysis induced by urokinase plus PLG. The fibrinolytic activity of urokinase was dramatically inhibited by either pleural leukocytes or their supernatants. The fibrinolytic activity of plasmin was less inhibitable than that of urokinase, with less than 40% of total fibrinolysis being blocked (Fig. 2.8). No inhibition of fibrinolysis induced by streptokinase plus PLG was seen after the addition of pleural leukocyte supernatants for up to 24 hr (Table 2.1).

Table 2.1 EFFECT OF PLEURAL LEUKOCYTE SUPERNATANT ON FIBRINOLYSIS INDUCED BY STREPTOKINASE PLUS PLG

Streptokinase (U/ml)	1	10	100
Dilution of supernatant			
1:8	42886(3111)	41071(5494)	33347(5563)
1:4	38949(3750)	35596(1074)	32505(3321)
1:2	36885(2222)	35122(1808)	35654(2734)

Results are presented as the mean(SD) cpm of 2 separate experiments in triplicate. No statistically significant difference was found between different concentrations of pleural leukocyte supernatants.

From these preliminary results, we believed that there existed a PA inhibitor(s) in the supernatant of pleural leukocytes. Because of the ability of the inhibitor to act mainly on urokinase plus PLG, it was presumed that the pleural leukocytes released a urokinase-type PAI. To substantiate this hypothesis further,

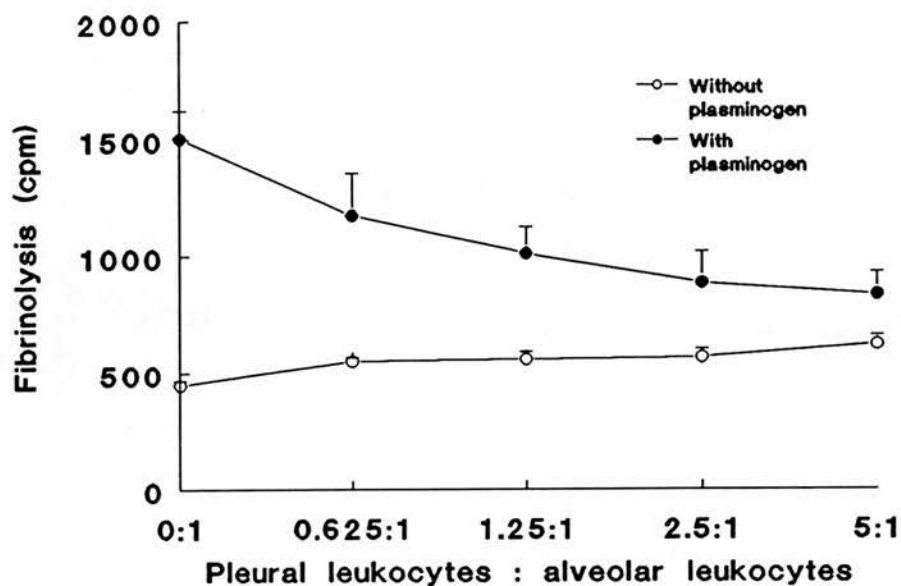


Figure 2.5 Effect of increasing concentrations of pleural leukocytes on alveolar leukocyte-induced fibrinolysis

Each point is mean (SEM) of triplicate wells in three separate experiments.

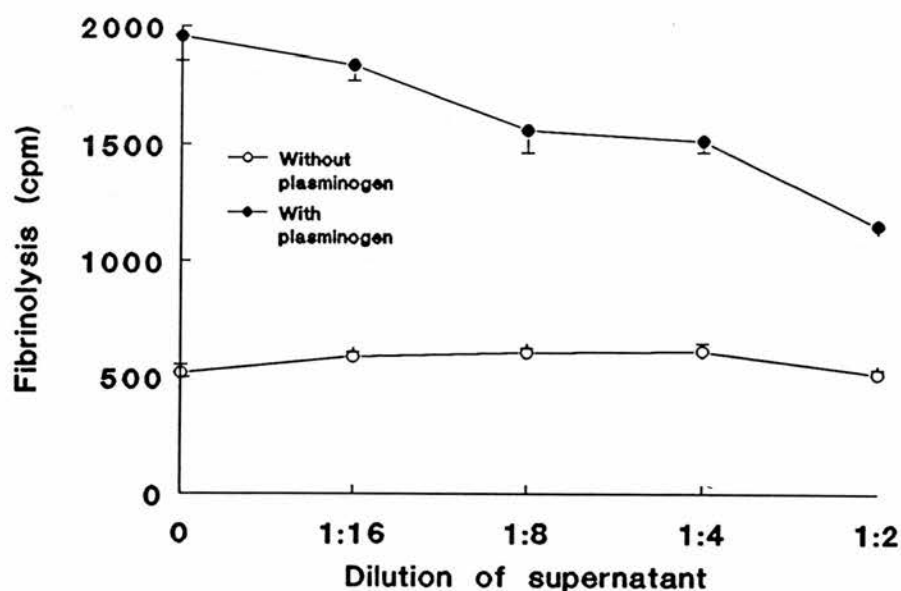


Figure 2.6 Effect of increasing concentrations of pleural leukocyte supernatant on alveolar leukocyte-induced fibrinolysis

Each point is mean (SEM) of triplicate wells in three separate experiments.

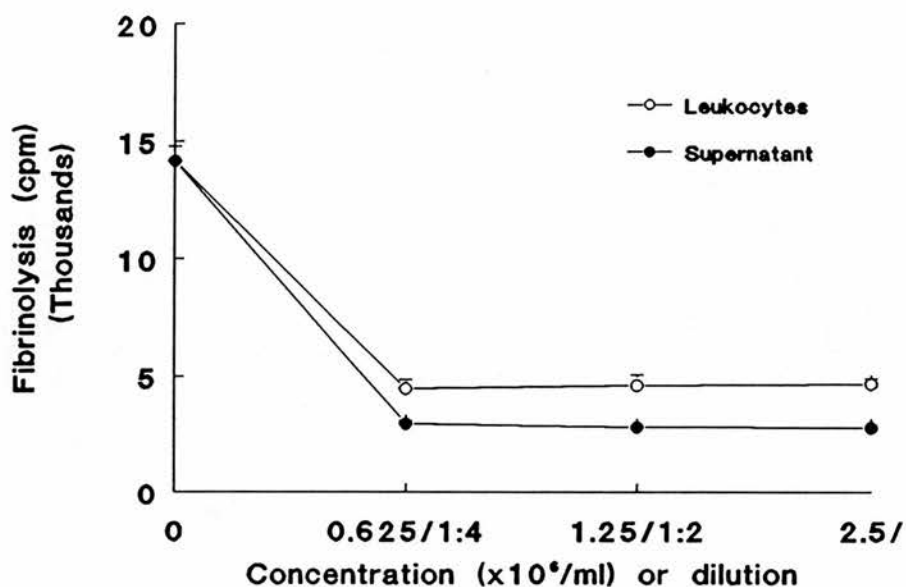


Figure 2.7 Effect of increasing concentrations of pleural leukocytes, or their supernatants, on fibrinolysis induced by urokinase plus plasminogen (PLG)

Each Point is mean (SEM) of triplicate wells in four separate experiments.

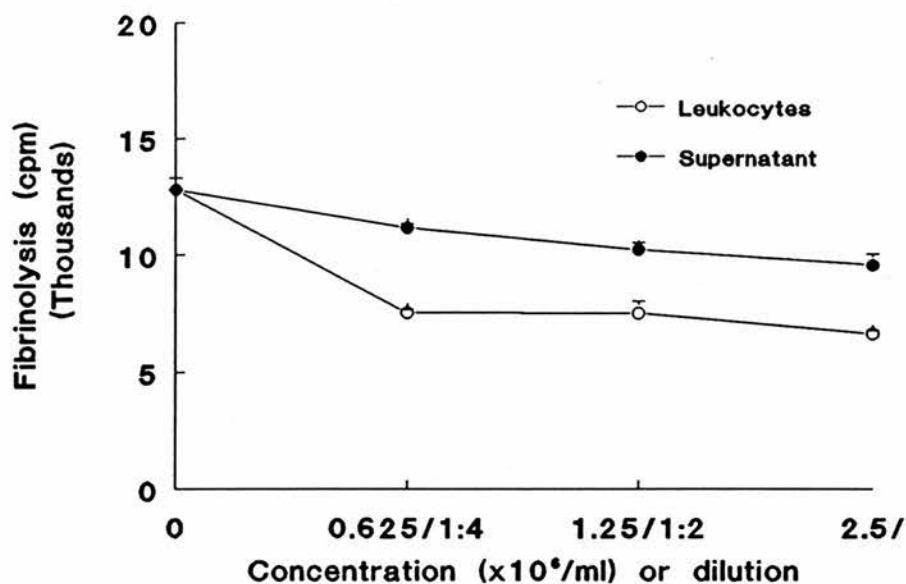


Figure 2.8 Effect of increasing concentrations of pleural leukocytes, or their supernatants, on plasmin-induced fibrinolysis

Each point is mean (SEM) of triplicate wells in four separate experiments.

some work to identify the cell source of PAI and to characterize the inhibitor was carried out using the urokinase plus PLG fibrinolysis system.

By using separation techniques, two subpopulations of pleural leukocytes were obtained: a macrophage-enriched population (93% macrophages) and a mast cell-enriched population (76% mast cells and 20% macrophages) (Table 2.2 and Figs 2.9, 2.10). The inhibition of fibrinolysis caused by supernatants from these subpopulations indicated that macrophages are by far the major source of PAI release in culture (Fig. 2.11). There was no significant difference between inhibition caused by whole pleural leukocytes and inhibition caused by the macrophage-enriched population. The fact that there was no increase in PAI activity corresponding to the increased proportion of macrophages in the macrophage-enriched subpopulation (from 82% to 93%) may be explained by slight damage caused to macrophages during the separation process.

Table 2.2 DIFFERENT CELL TYPES PRESENT IN RAT PLEURAL LEUKOCYTE SUBPOPULATIONS AFTER SEPARATION

	Differential percentage			
	Macrophages	Lymphocytes	Mast-cells	Eosinophils
Macrophage-enriched population	93(3.5) ^a	4(1.5)	2(2.3)	1(1.2)
Mast cell-enriched population	20(12.4) ^b	0	76(12.8)	4(1.4)

^a_n=8 rats, ^b_n=4 rats. Each result is shown as mean(SD).

In these experiments, a certain level of fibrinolysis was obtained with urokinase plus PLG such that PA could be detected by enhanced fibrinolysis and PAI could also be detected as reduced fibrinolysis. Studies with lysates indicated that pleural leukocytes contain high PA activity intracellularly (Fig. 2.12). This activity seems to be contained mainly inside mast cells, with very limited intracellular PA activity in macrophages.

The inhibitory activity of supernatants from pleural leukocytes remained constant after incubation at 56°C for 1 hr but was virtually abolished after incubation at 70°C for 1 hr (Fig. 2.13). The supernatant of pleural leukocytes was also assayed for fibrinolytic activity after being fractionated into 2 parts: molecular greater and less than 10,000 daltons. The fibrinolytic activity of urokinase plus PLG was inhibited by the high-molecular weight (MW) fraction,

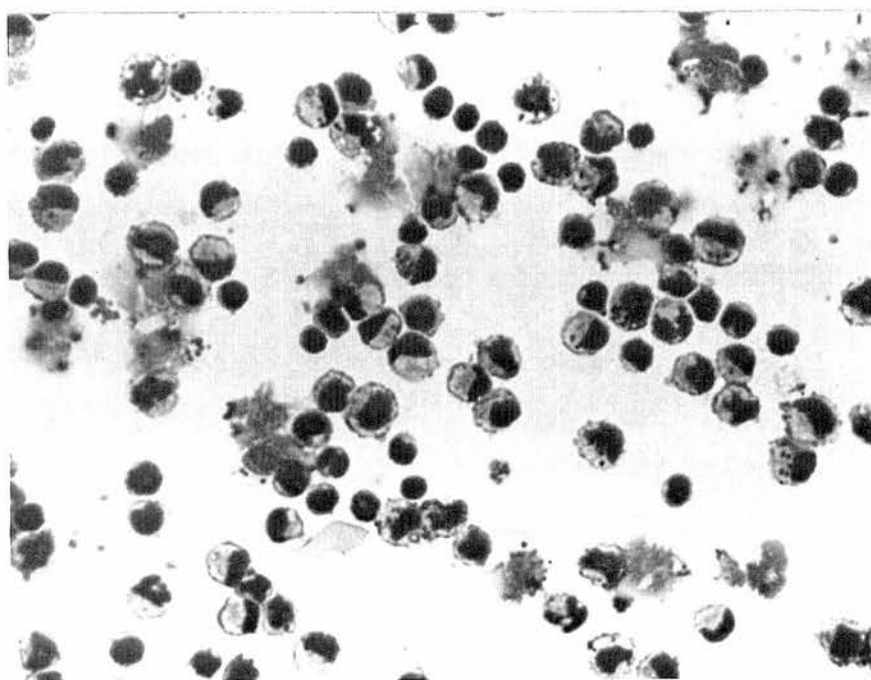


Figure 2.9 Light micrograph of macrophage-enriched pleural leukocytes (x200)

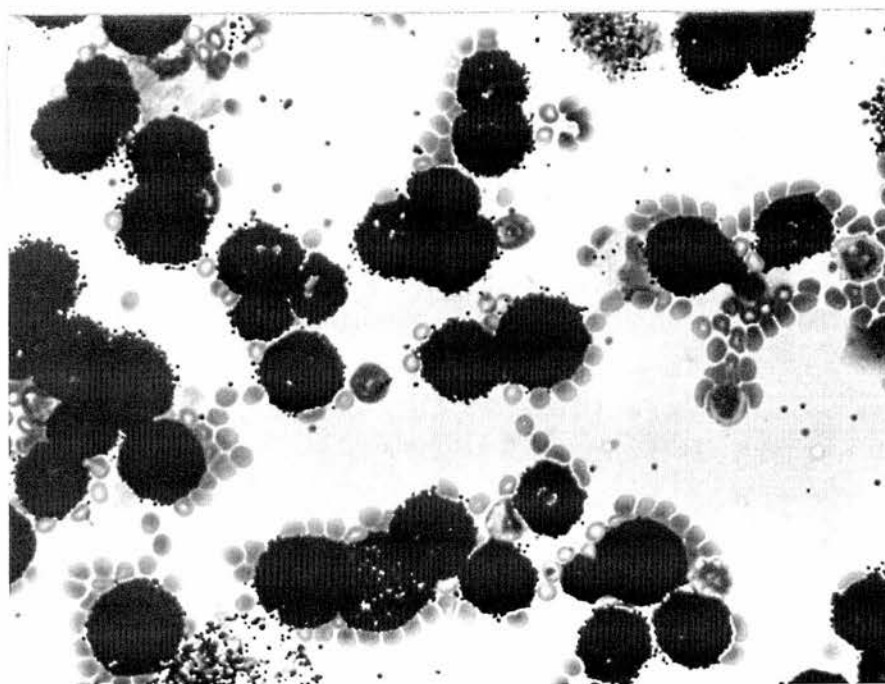


Figure 2.10 Light micrograph of mast cell-enriched pleural leukocytes (x200)

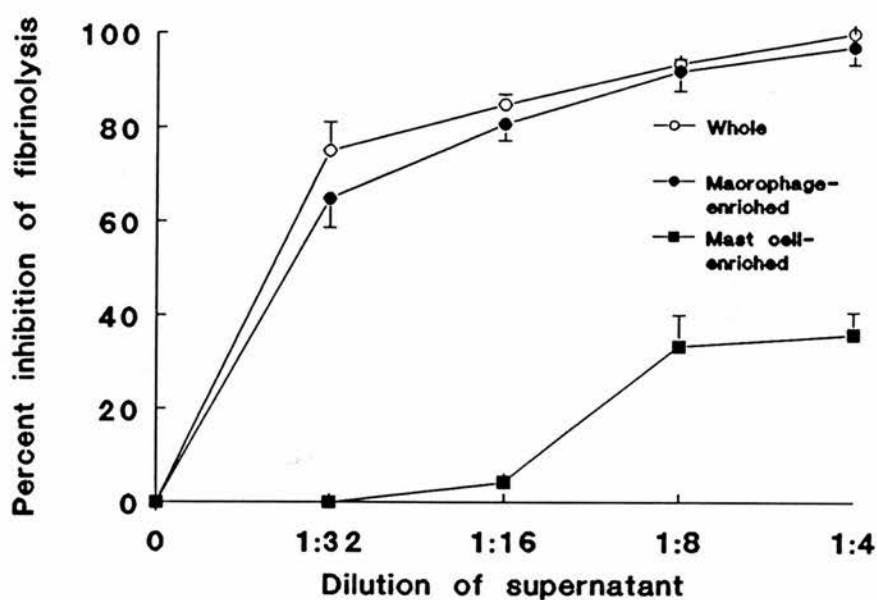


Figure 2.11 Inhibition of urokinase plus PLG-induced fibrinolysis by supernatants of separated subpopulations of pleural leukocytes

Each point represents mean (SEM) of triplicate wells in two separate experiments.

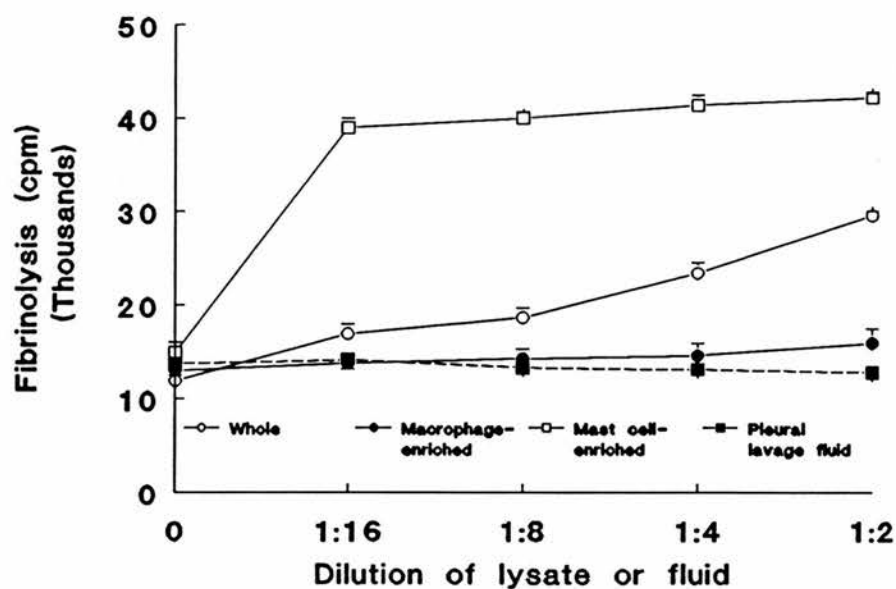


Figure 2.12 Effects of lysates of separated subpopulations of pleural leukocytes, or pleural lavage fluid, on fibrinolysis induced by urokinase plus PLG

Each point stands for mean (SEM) of triplicate wells in three separate experiments.

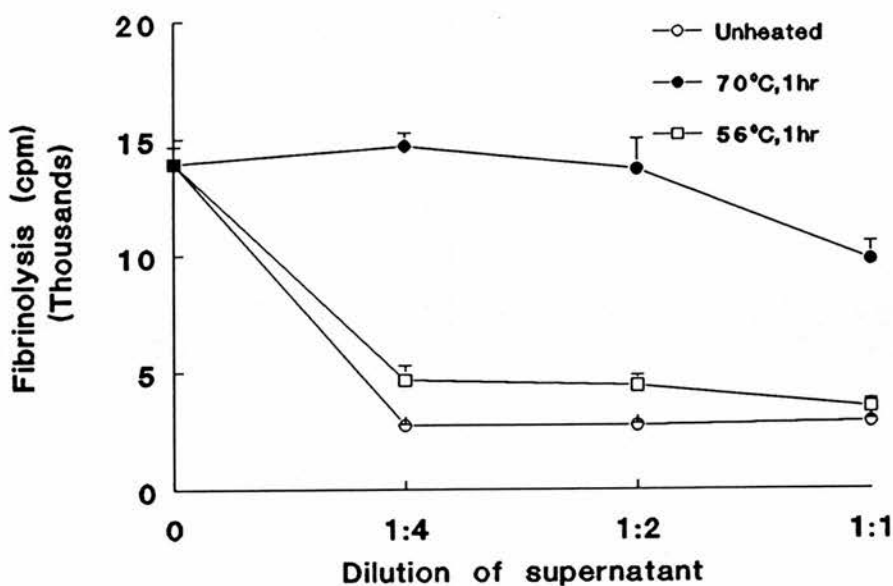


Figure 2.13 Inhibitory effects of heat-treated pleural leukocyte supernatants on fibrinolysis induced by urokinase plus PLG

Each point represents mean (SEM) of triplicate wells in three separate experiments.

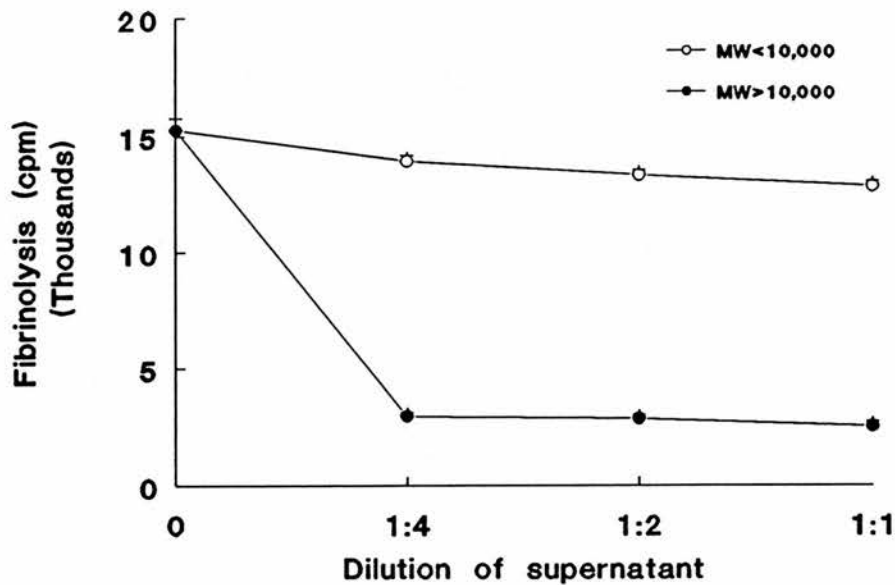


Figure 2.14 Inhibitory effects of different fractions of pleural leukocyte supernatant on fibrinolysis induced by urokinase plus PLG

Each point represents mean (SEM) of triplicate wells in two separate experiments.

while no inhibitory effect could be observed in the low-MW fraction (Fig. 2.14). Acid treatment of the inhibitor revealed that the inhibitor was acid labile (Fig. 2.15). These results provided evidence that the inhibitor of urokinase in supernatants of pleural leukocytes is a high-MW, heat and acid labile substance, probably a protein. Moreover, it was found that stimulated with LPS, pleural leukocytes did not produce increased PAI activity in culture than non-stimulated pleural leukocytes (Table 2.3)

Table 2.3 THE EFFECT OF LPS STIMULATION ON PAI PRODUCTION BY PLEURAL LEUKOCYTES

Dilution of supernatant	% inhibition of fibrinolysis	
	With LPS	Without LPS
1:32	30.62 (8.16)	28.13 (11.14)
1:16	50.23 (13.95)	48.35 (12.83)
1:8	72.38 (14.2)	76.70 (7.80)
1:4	90.66 (5.08)	97.12 (3.02)

Results are presented as the mean(SD) of percent inhibition of urokinase-induced fibrinolysis from 3 separate experiments in triplicate.

2.2.3. Increased production of PAI by pleural leukocytes from rats intratracheally instilled with crocidolite asbestos

As reported above, normal pleural leukocytes do not express any PA activity in culture but, intracellularly, pleural leukocytes, mainly mast cells, contain large quantities of PA activity. After intratracheal instillation of crocidolite asbestos, there was no PA activity detectable in asbestos-exposed pleural leukocyte supernatants. There was also no significant difference in PA activity in cell lysates of control pleural leukocytes and leukocytes one day after 10 mg crocidolite I/T (Table 2.4).

Two controls, 0.5 ml PBS I/T and complete normal controls, were compared and the PAI secretion by the leukocytes from these two control rats showed no significant difference (Table 2.5).

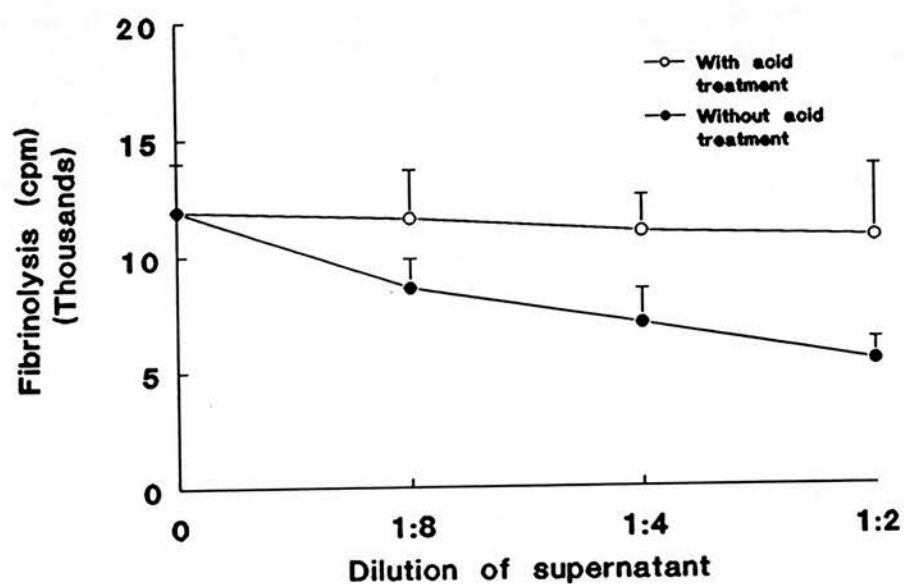


Figure 2.15 Effect of acid-treatment on the activity of PAI in pleural leukocyte supernatant

Results are presented as mean and SEM of 5-7 experiments in triplicate.

Table 2.4 PA ACTIVITY IN THE LYSATES OF PLEURAL LEUKOCYTES FROM CONTROL AND ASBESTOS INSTILLED RATS

Treatment	Control	Crocidolite I/T
Dilution of lysate		
1:32	18581(1882)	16795(1619)
1:16	17464(3823)	16307(2802)
1:8	20530(4530)	18975(3903)
1:4	21878(2500)	21749(4302)
1:2	31494(3177)	28087(2156)

Results are presented as the mean(SD) cpm of 2 separate experiments in triplicate. No statistical significant difference was found between the two treatment groups. Crocidolite I/T: 1 day after 10 mg crocidolite given by intratracheal instillation.

Table 2.5 PLASMINOGEN ACTIVATOR INHIBITOR SECRETION BY PLEURAL LEUKOCYTES FROM UNTREATED AND PBS-TREATED RATS

Dilution	PBS I/T	Normal
1:32	59.81 (19.58)	62.94 (6.22)
1:16	73.15 (15.39)	79.32 (2.81)
1:8	78.99 (14.18)	83.79 (5.00)
1:4	94.41 (4.00)	96.92 (2.14)

Results are presented as mean(SD) of percent inhibition of urokinase-induced fibrinolysis by pleural leukocyte supernatants, n=6 rats for both groups. There was no significant difference between two groups.

However, as shown in Fig. 2.16, the percent inhibition of urokinase activity caused by pleural leukocytes from asbestos- exposed rats was significantly greater than controls. At almost all of the time points following exposure to asbestos, supernatants exhibited markedly increased PAI activity. Nevertheless, there was no significant difference in the production of inhibitor between different time points following asbestos instillation. The apparent difference in Fig. 2.16 was masked by the variation between experiments.

Figure 2.17 shows the inhibition of urokinase-induced fibrinolysis caused by

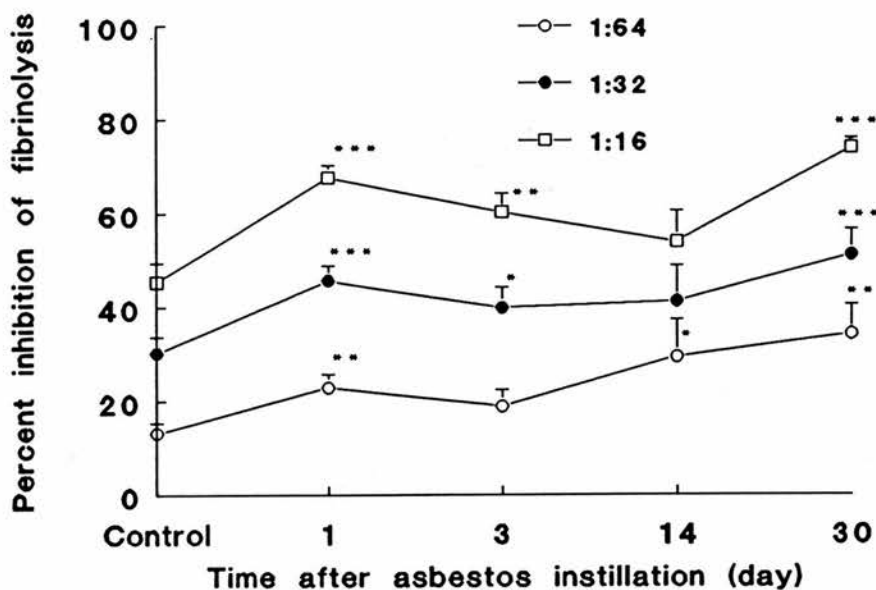


Figure 2.16 Inhibition of urokinase plus PLG-induced fibrinolysis caused by supernatants of pleural leukocytes from rats at various times after I/T 5 mg crocidolite asbestos

Each point represents the mean and SEM of triplicate wells in 3-14 separate experiments. Asterisk denotes a significant difference from corresponding dilution of control: * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$.

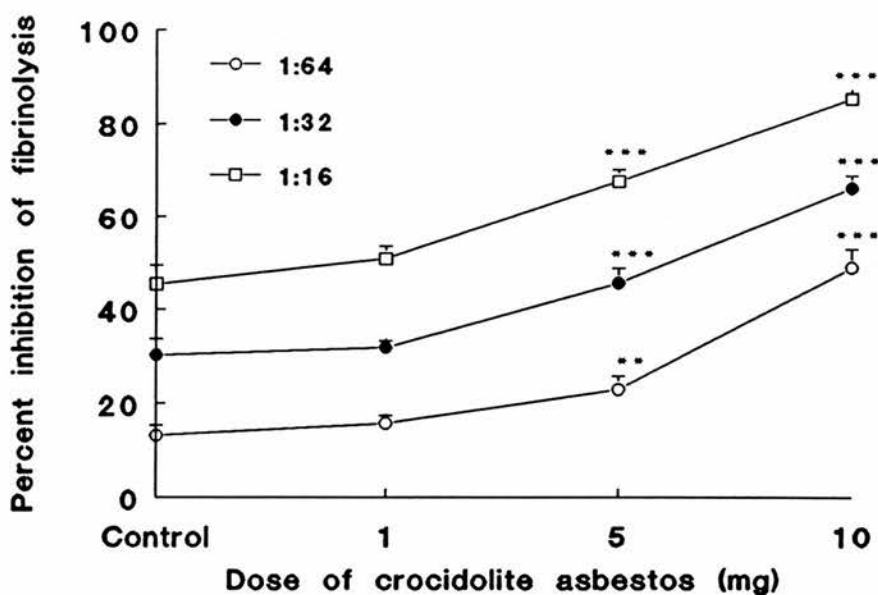


Figure 2.17 Inhibition of urokinase plus PLG-induced fibrinolysis by supernatants of pleural leukocytes from rats one day after I/T different doses of crocidolite asbestos

Each point stands for the mean and SEM of triplicate wells in 5-14 separate experiments. Asterisk denotes a significant difference from corresponding dilution of control: ** = $P < 0.01$ and *** = $P < 0.001$.

supernatants of pleural leukocytes from rats one day after various doses of asbestos I/T. It was very clear that asbestos stimulated secretion of PAI by pleural leukocytes in a dose-related manner, although the cells recovered from rats dosed with 1 mg asbestos did not express significantly higher PAI activity in culture than controls.

2.3. Interleukin-1 (IL-1) production by rat pleural leukocytes in culture after I/T crocidolite asbestos

Normal pleural leukocytes produced IL-1 in culture, even in the absence of endotoxin stimulation (Fig. 2.18). The activity of IL-1 in supernatants of normal leukocytes without stimulation was about 167 U/ml compared to IL-1 β standard. After 100 ng/ml of LPS stimulation, the activity of IL-1 by pleural leukocytes reached approximately double the normal levels, to about 268 U/ml. At the highest concentration of LPS-stimulated supernatant (1:4), the abnormal reduction of IL-1 activity might be due to too high concentration in IL-1 in supernatant which inhibits the mitogenesis of C3H mouse thymocytes.

Five mg crocidolite asbestos I/T resulted in a decrease in the IL-1 activity of pleural leukocyte supernatants as demonstrated by Fig. 2.19. One day after ⁵/₁ 5 mg crocidolite, the IL-1 activity decreased most significantly. With increasing time after instillation, the IL-1 activity recovered towards normal levels. Moreover, IL-1 production was increased over control levels by 30 days after I/T asbestos.

Figure 2.20 illustrates the dose-related effect of crocidolite I/T on IL-1 elaboration by pleural leukocytes in culture. For all asbestos-treated leukocyte supernatants, IL-1 activity decreased and this decrease was correlated with the increase of the dose of instilled asbestos.

2.4. Crocidolite asbestos I/T depresses the high secretion of tumor necrosis factor (TNF) by pleural leukocytes in culture

As shown in Figure 2.21, normal pleural leukocytes secreted a high level of TNF in culture in the absence of LPS. The concentration of TNF in supernatants of normal leukocytes was about 648 U/ml using recombinant TNF α as a standard. There was no significant difference between two different controls, i.e., completely normal rats and saline I/T rats. The presence of 100 ng/ml of LPS enhanced the TNF production by several times (about 1760 U/ml) compared to the constitutive release.

Up to 30 days after 5 mg crocidolite asbestos I/T, the capacity of pleural leukocytes to secrete TNF in culture was reduced significantly compared to controls. There was, however, no detectable time-related effect in this reduction of secretion with TNF activity present at 1 day being similar to that found at 1

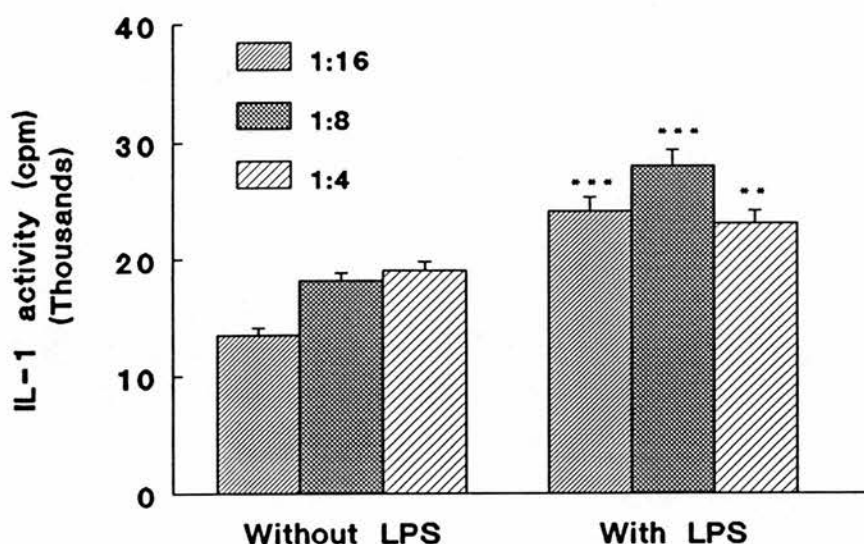


Figure 2.18 IL-1 production by normal pleural leukocytes with or without simulation with LPS

Results are presented as mean and SEM of 10-14 separate experiments performed in triplicate. The concentration of LPS used in the study was 100 ng/ml. Asterisk denotes a significant difference from corresponding dilution of control: **= $P < 0.01$ and ***= $P < 0.001$.

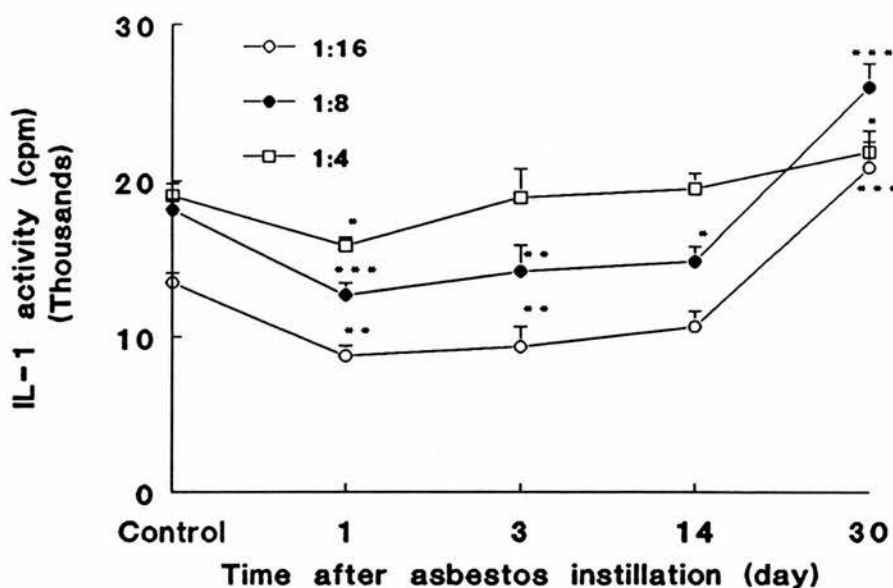


Figure 2.19 IL-1 release by pleural leukocytes from rats at various times after I/T 5 mg crocidolite asbestos

Each point represents the mean and SEM of triplicate wells in 9-15 separate experiments. Asterisk denotes a significant difference from corresponding dilution of control: *= $P < 0.05$, **= $P < 0.01$ and ***= $P < 0.001$.

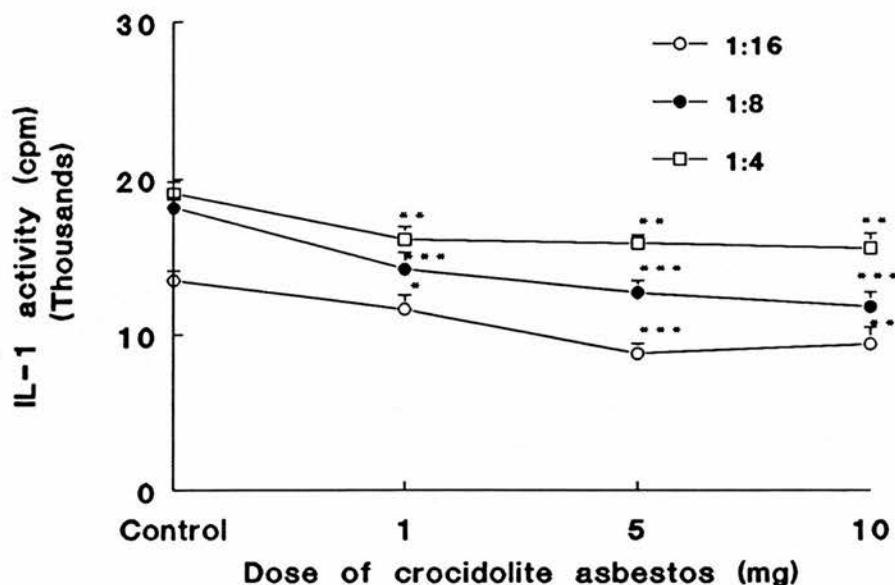


Figure 2.20 IL-1 release by pleural leukocytes from rats one day after I/T various doses of crocidolite asbestos

Each point represents the mean and SEM of triplicate wells in 9-16 separate experiments. Asterisk denotes a significant difference from corresponding dilution of control: *= $P < 0.05$, **= $P < 0.01$ and ***= $P < 0.001$.

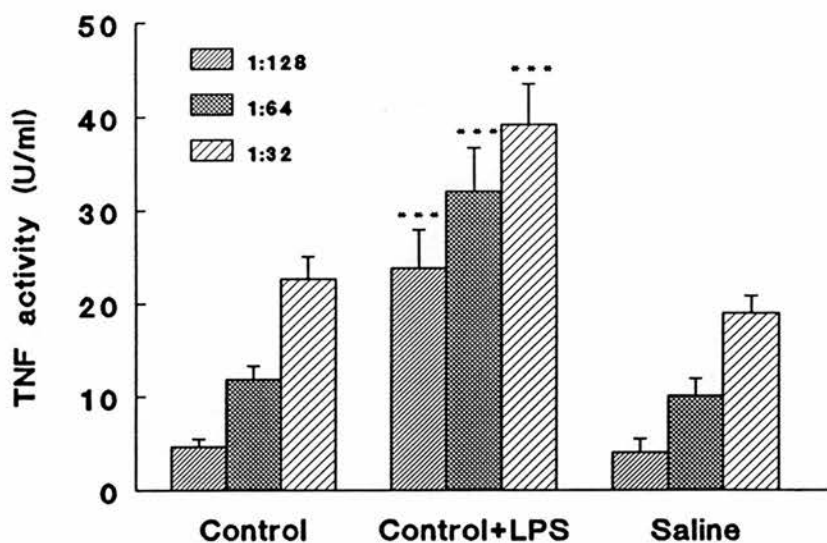


Figure 2.21 TNF production by normal pleural leukocytes with or without stimulation with LPS and by pleural leukocytes from I/T saline control

Each point represents the mean and SEM of triplicate wells in 9-22 separate experiments. Asterisk denotes a significant difference from corresponding dilution of control: ***= $P < 0.001$.

month (Fig. 2.22).

Fig. 2.23 illustrates that one day after intratracheal instillation, increasing doses of crocidolite in the lung caused a gradual decrease in the ability of the pleural leukocytes to release TNF *in vitro*; this was particularly notable for 10 mg crocidolite group, where TNF secretion was inhibited almost completely.

2.5. Effects of anti-TNF serum on TNF activity in pleural leukocyte supernatants

Anti-TNF serum was found to inhibit the activity of the TNF α standard. Figure 2.24 shows a clear inhibitory effect of rabbit anti-TNF serum on TNF α standard in a concentration-related manner.

Anti-TNF serum also had an inhibitory effect on TNF activity in pleural leukocyte supernatants. In contrast, control serum had no such inhibitory effect (Fig. 2.25).

2.6. Determination of contaminating endotoxin level in the media used in the studies

To ascertain the relationship between contaminating endotoxin in the media and spontaneous release of cytokines in the present studies, endotoxin levels were determined. The activity, in pg/ml of media were as following:- distilled water 0, saline 0, PBS 16, MEM + 5% FCS 29, F10 + 2% BSA 106, RPMI + 10% FCS 0, F12 + 10% FCS 46, the levels in the prepared supernatants were 98-112. TNF activities in pleural leukocytes supernatants which were prepared in medium before or after endotoxin removal by polymyxin columns showed no significant difference: TNF activities (mean(SD)n, U/ml calculated from a supernatant dilution of 1:16):- normal medium 1086(172)12; endotoxin-depleted medium 1063(81)12.

3. Alveolar leukocyte responses to intratracheal instillation of crocidolite asbestos

In this section, all experiments were carried out using HAN rats due to the shortage of supply of PVG rats.

3.1. The effect of crocidolite asbestos I/T on the bronchoalveolar leukocyte populations

Table 3.1 shows the distribution of different components in alveolar leukocyte population various times following 5 mg crocidolite asbestos I/T. It was difficult to perform bronchoalveolar lavage sometimes due presumably to the blockage of airways by instilled fibres, so that the total cell numbers in the lavage were not accurate. The results were therefore presented as percentages of cells rather than total numbers.

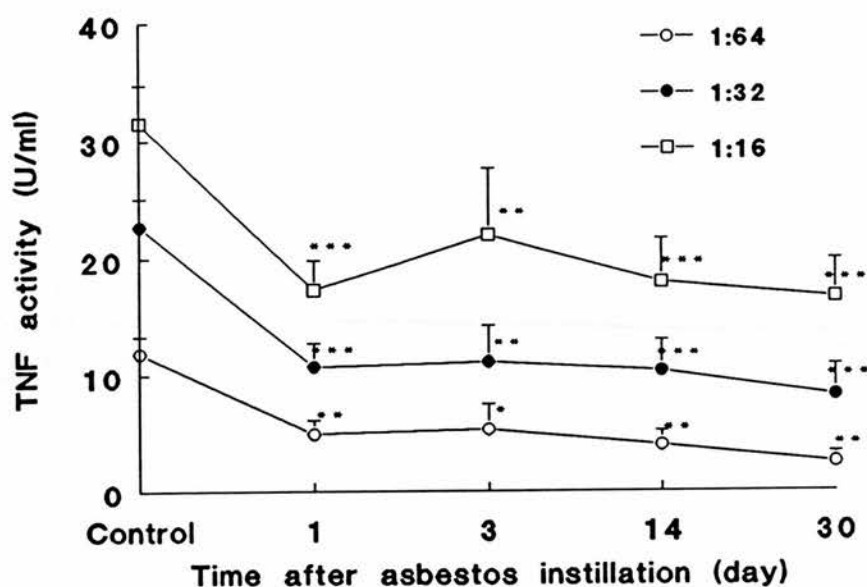


Figure 2.22 TNF production by pleural leukocytes from rats at various times after I/T 5 mg crocidolite asbestos

Each point represents the mean and SEM of triplicate wells in 10–22 separate experiments. Asterisk denotes a significant difference from corresponding dilution of control: *= $P < 0.05$, **= $P < 0.01$ and ***= $P < 0.001$.

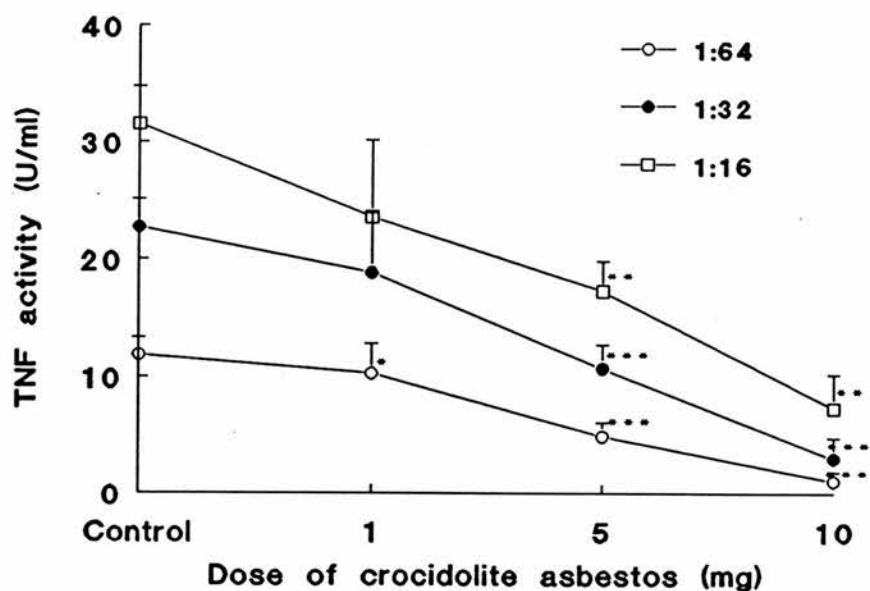


Figure 2.23 TNF production by pleural leukocytes from rats one day after I/T various doses of crocidolite asbestos

Each point represents the mean and SEM of triplicate wells of 9–22 separate experiments. Asterisk denotes a significant difference from corresponding dilution of control: *= $P < 0.05$, **= $P < 0.01$ and ***= $P < 0.001$.

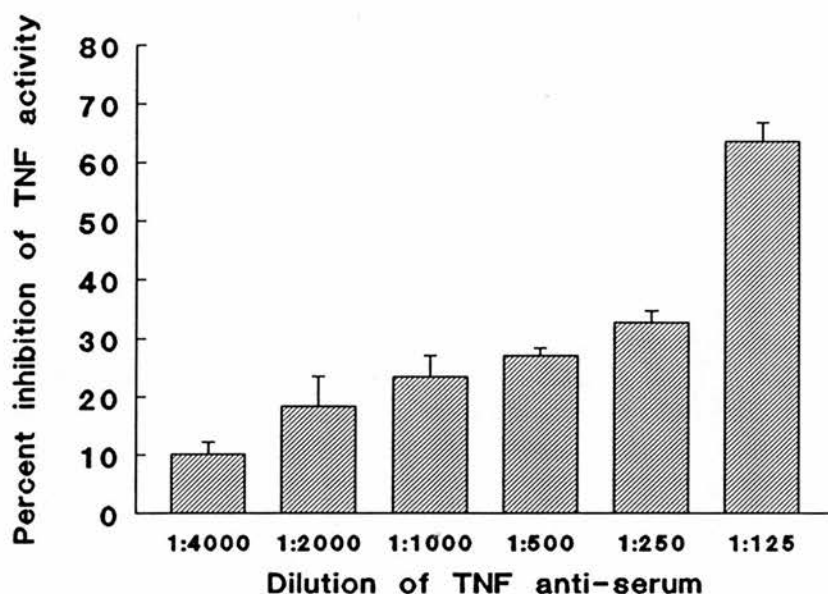


Figure 2.24 Percent inhibition on the activity of $\text{TNF}\alpha$ standard caused by anti-TNF serum

Each bar stands for mean and SEM of one experiment in triplicate.

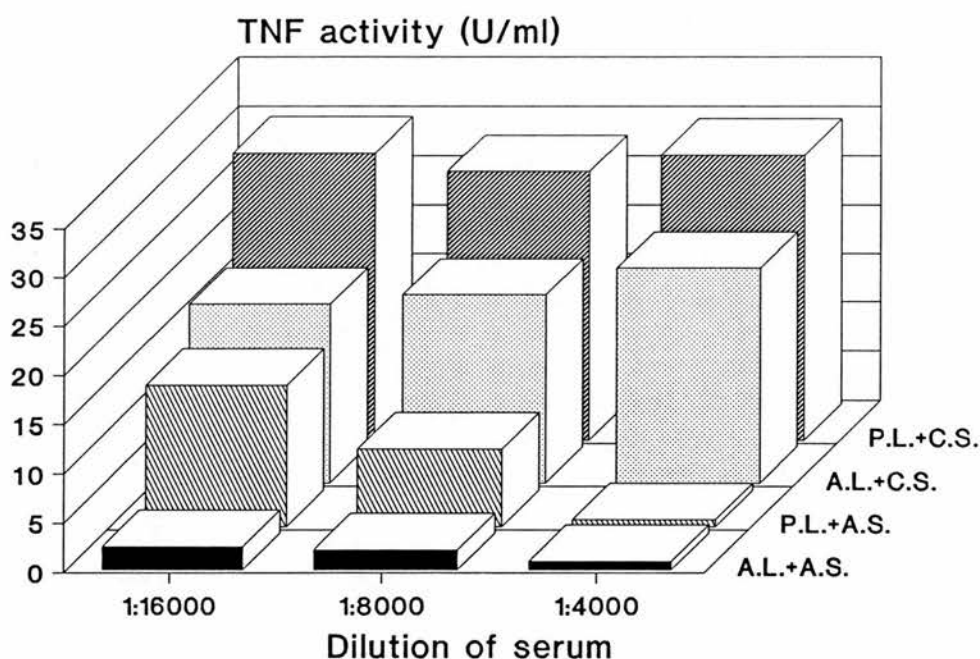


Figure 2.25 Effect of control and anti-TNF sera on TNF activity in supernatants of normal pleural and bronchoalveolar leukocytes

Each bar represents the mean of 3–6 separate experiments in triplicate. The dilution of supernatants was 1:16. SEM, varies from 12 to 86% of mean, was not shown on the graph. P.L.:– pleural leukocyte supernatant; A.L.:– alveolar leukocyte supernatant; C.S.:– control serum and A.S.:– anti-TNF serum.

Table 3.1 BRONCHOALVEOLAR LEUKOCYTE COMPONENTS VARIOUS TIMES AFTER 5 mg CROCIDOLITE ASBESTOS I/T

Time (Day)	Total number ($\times 10^6$)	Differential percentage			
		Macrophages	PMN	Lymphocytes	Eosinophils
Control	5.0(2.7)	99(0.7)	0	1(0.8)	0
1	2.7(2.5)	64(35.5)	26(39.9)	1(0.7)	9(8.9)
3	1.8(1.2)	70(12.4)	14(8.4)	1(0.7)	15(8.3)
14	2.4(1.9)	95(1.3)	3(1.4)	1(0.6)	1(0.8)
30	3.1(2.0)	95(6.4)	4(5.5)	1(0.8)	0

n=7-12 rats. Results are presented as mean(SD).

It was clear that the percentage of macrophages decreased dramatically 1 day after asbestos instillation due to the marked increase of PMN in the alveolar space at the same time. Meanwhile, the percentage of eosinophils increased although this cell component was hardly seen in normal bronchoalveolar leukocyte populations. From three days after asbestos I/T, the percentage of macrophages went up slightly and was restored almost completely 2 weeks after instillation. This corresponded to the rapid decrease of the percentage of PMN in the alveolar spaces from 3 days after asbestos I/T. Similar to the pattern with PMN, the percentages of eosinophils remained at higher levels during the first three days after instillation and then waned.

The changes of cell components of bronchoalveolar leukocytes one month after various doses of crocidolite asbestos I/T are shown in Table 3.2. In comparison to control, most cell components remained almost at the same level except for a moderate increase in the percentage of PMN in the asbestos-exposed group. The total cell numbers increased markedly compared to control, although the 5 mg group had less numbers which may result from lavage difficulty.

Table 3.2 BRONCHOALVEOLAR LEUKOCYTE COMPONENTS ONE MONTH AFTER I/T VARIOUS DOSES OF CROCIDOLITE ASBESTOS

dose (mg)	Total number ($\times 10^6$)	Differential percentage			
		Macrophages	PMN	Lymphocytes	Eosinophils
Control	5.0(2.7)	99(0.7)	0	1(0.8)	0
0.5	8.8(3.6)	94(6.9)	5(6.7)	1(0.4)	0
1	10.6(5.5)	97(1.9)	2(1.5)	1(0.5)	0
5	3.1(2.0)	95(6.4)	4(5.5)	1(0.8)	0

n=6-12 rats. Results are presented as mean(SD). Compared to control, total cell numbers of 0.5 and 1 mg groups were significant different ($P<0.05$).

3.2. IL-1 production by bronchoalveolar leukocytes from rats various times after I/T 5 mg crocidolite asbestos

Figure 3.1 shows that normal alveolar leukocytes produced IL-1 at about 36 U/ml in culture without added stimulation compared to IL-1 β standard, which was much less than IL-1 activity produced by normal pleural leukocytes (Fig.2.18). Intratracheal instillation of crocidolite asbestos caused decrease of IL-1 elaboration by bronchoalveolar leukocytes in culture. From 1 day to 14 days after instillation, the IL-1 activity in the leukocyte supernatants decreased dramatically compared to control supernatants. However, when it was one month after asbestos instillation, IL-1 production by alveolar leukocytes increased considerably and was beyond the level of control leukocytes.

The effect of various doses of crocidolite I/T on IL-1 production by alveolar leukocytes was presented in Fig. 3.2. A dose-related effect of instilled asbestos on alveolar leukocytes was seen in IL-1 production.

3.3. Increased production of TNF by bronchoalveolar leukocytes after rats were intratracheally instilled with crocidolite asbestos

Figure 3.3 shows that normal alveolar leukocytes released TNF spontaneously at the level of 561 U/ml which was slightly lower than that released by normal pleural leukocytes. Crocidolite asbestos I/T caused bronchoalveolar leukocytes to produce more TNF in culture than control at various times except one day post instillation. However, there was no significant effect of time.

As shown in Fig. 3.4, although 0.5 mg asbestos I/T seemed not to be able to stimulate alveolar leukocytes, 1 or 5 mg asbestos caused increased production of TNF by alveolar leukocytes with a dose-response pattern.

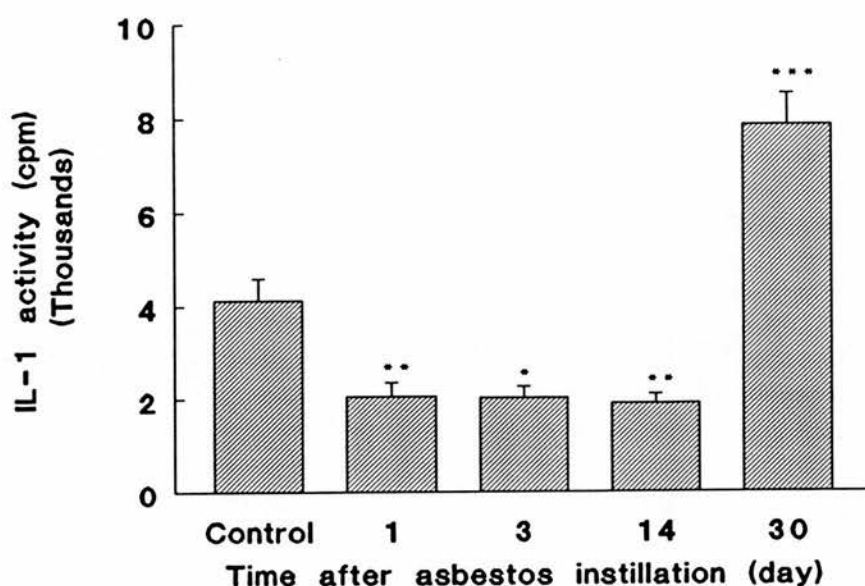


Figure 3.1 IL-1 production by alveolar leukocytes from rats at various time after I/T 5 mg crocidolite asbestos

Each bar represents the mean and SEM of triplicate wells in 4-11 separate experiments. Asterisk denotes a significant difference from control: *= $P < 0.05$, **= $P < 0.01$ and ***= $P < 0.001$. The dilution of supernatant was 1:4.

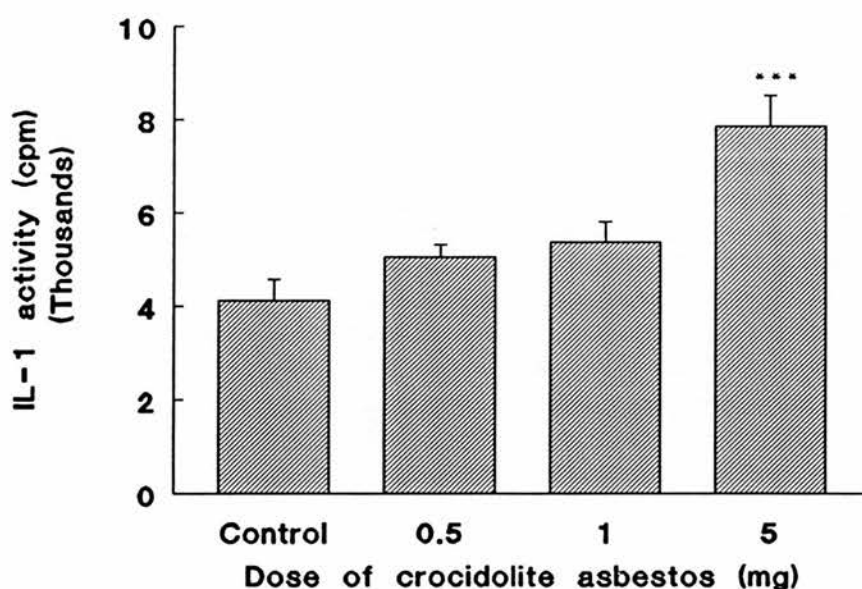


Figure 3.2 IL-1 production by alveolar leukocytes from rats one month after I/T various doses of crocidolite asbestos

Each bar represents the mean and SEM of triplicate wells in 6-11 separate experiments. Asterisk denotes a significant difference from control: ***= $P < 0.001$. The dilution of supernatant was 1:4.

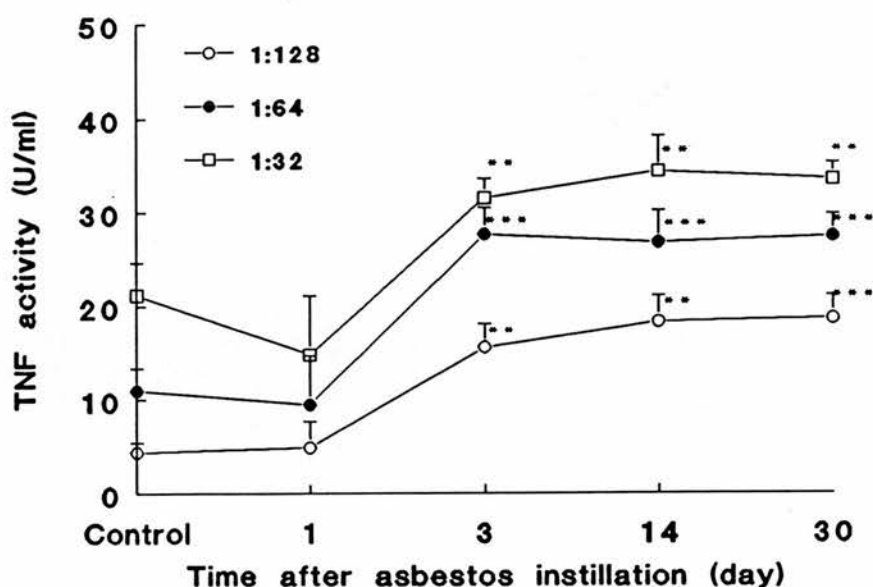


Figure 3.3 TNF production by alveolar leukocytes from rats at various times after I/T 5 mg crocidolite asbestos

Each point represents the mean and SEM of triplicate wells in 5–15 separate experiments. Asterisk denotes a significant difference from corresponding dilution of control: ** = P < 0.01 and *** = P < 0.001.

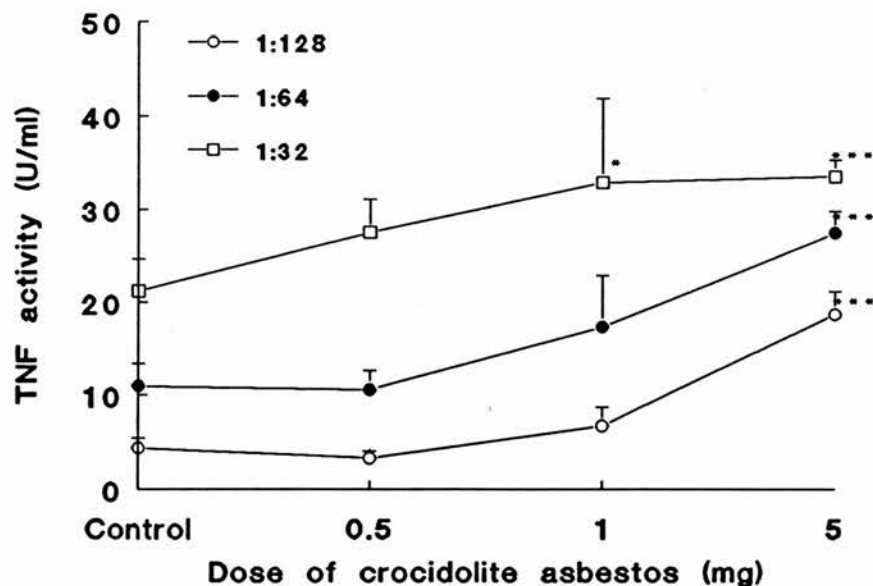


Figure 3.4 TNF production by alveolar leukocytes from rats one month after I/T different doses of crocidolite asbestos

Each point represents the mean and SEM of triplicate wells in 5–15 separate experiments. Asterisk denotes a significant difference from corresponding dilution of control: * = P < 0.05 and *** = P < 0.001.

The specificity of TNF activity in alveolar leukocyte supernatants was also tested using rabbit anti-TNF serum and control serum. Results indicated that TNF activity in the supernatants was inhibitable by anti-TNF serum and not by control serum (Figs. 2.25).

4. Studies on combined effects of crocidolite and other dusts on pleural and bronchoalveolar leukocytes

In this section, all of experiments were carried out using HAN rats.

4.1. In *in vivo* studies on combined effects of crocidolite and other dusts I/T on bronchoalveolar and pleural leukocytes

4.1.1. Cellular changes in the bronchoalveolar and pleural spaces after crocidolite and/or other dusts I/T

Table 4.1 shows the components of bronchoalveolar leukocytes one month after 2.5 mg dusts I/T individually or in combination. When dusts were instilled individually, TiO_2 and crocidolite did not cause important changes in alveolar leukocyte components, whereas quartz (DQ_{12}) resulted in massive recruitment of leukocytes with a high percentage of PMN. A synergistic effect on total number of leukocytes was observed with crocidolite plus DQ_{12} . The slight decrease in total number in the asbestos-treated groups was considered to be due to lavage difficulties caused by airway blockage by asbestos fibres.

BRONCHOALVEOLAR LEUKOCYTE COMPONENTS ONE MONTH AFTER I/T INDIVIDUALLY OR IN COMBINATION

Dust	Total No. ($\times 10^6$)	Differential Percentage		
		Macrophage	PMN	Lymphocyte
Control	4.98(2.67)	98.86(0.69)	0.14(0.38)	1.00(0.82)
TiO_2	3.82(0.55)	98.33(1.15)	0.67(1.15)	1.00(0)
DQ_{12}	26.27(3.38)	57.33(18.50)	42.00(18.52)	0.67(0.58)
Cro	2.43(0.62)	97.67(1.53)	1.33(1.53)	1.00(0)
Cro+ TiO_2	3.57(0.53)	96.67(1.15)	3.33(1.15)	0(0)
Cro+ DQ_{12}	43.27(11.15)	72.00(5.29)	27.00(5.29)	1(0)

n=9 rats for controls and 3 rats for all other treatment. Results are presented as mean(SD). Cro:-crocidolite asbestos. There is a statistically significant difference between control v DQ_{12} ($P<0.001$), control v cro+ DQ_{12} ($P<0.001$) and DQ_{12} v cro+ DQ_{12} ($P<0.05$).

The components of the pleural leukocytes after I/T asbestos and/or other

dusts are presented in Table 4.2. Instillation of dusts caused an increase in total numbers. In the crocidolite plus DQ₁₂ group, the total number increased most dramatically. The percentages of macrophages decreased with combinations of crocidolite plus TiO₂ or DQ₁₂, whilst that of eosinophils increased.

Table 4.2 PLEURAL LEUKOCYTE COMPONENTS ONE MONTH AFTER DUSTS I/T INDIVIDUALLY OR IN COMBINATION

Dust	Total number		Differential percentage		
	($\times 10^6$)	Macrophages	Lymphocytes	Mast-cells	Eosinophils
Control	4.5(1.0)	82(5.6)	1(1.2)	11(5.4)	1(0.8)
TiO ₂	10.8(2.3)	84(2.3)	1(1.0)	8(1.2)	7(3.0)
DQ ₁₂	9.6(1.7)	89(0.7)	0	5(1.4)	6(0.7)
Cro	11.6(2.5)	82(1.2)	1(1.0)	10(1.2)	7(2.1)
Cro+TiO ₂	12.5(2.3)	77(7.2)	1(0.6)	10(6.2)	12(5.2)
Cro+DQ ₁₂	19.6(2.3)	79(2.1)	2(1.5)	6(2.0)	13(2.7)

n=4 rats for control and 3 rats for all other treatment groups. No PMN was seen in all of groups. Results are presented as mean (SD). All dusts groups are significantly higher than control groups ($P<0.001$). Cro v cro+DQ₁₂ is also significantly different ($P<0.01$).

4.1.2. TNF and IL-1 production by bronchoalveolar and pleural leukocytes one month after 2.5 mg dusts I/T individually or in combination

Figure 4.1 shows that in comparison to TNF level produced by control alveolar leukocytes, TiO₂ stimulated alveolar leukocytes produced considerably increased TNF activity, whereas DQ₁₂ I/T lead to decreased TNF production by the cells. Crocidolite asbestos did not affect the production. No notable interaction on TNF production resulted from the combination of crocidolite asbestos and other dusts.

In the pleural space, there was a totally different picture. Fig. 4.2 illustrates that individually all of the dusts caused a significant reduction of TNF production by pleural leukocytes. The combination of crocidolite asbestos with other dusts, however, stimulated a dramatic increase of TNF release by the cells with a considerable synergetic effect. When crocidolite was instilled together with DQ₁₂, their stimulating effect increased nearly 5-fold compared to control. Even when crocidolite was combined with TiO₂ which is considered an inert dust, they stimulated pleural leukocytes to produce 2-fold more TNF in culture than control.

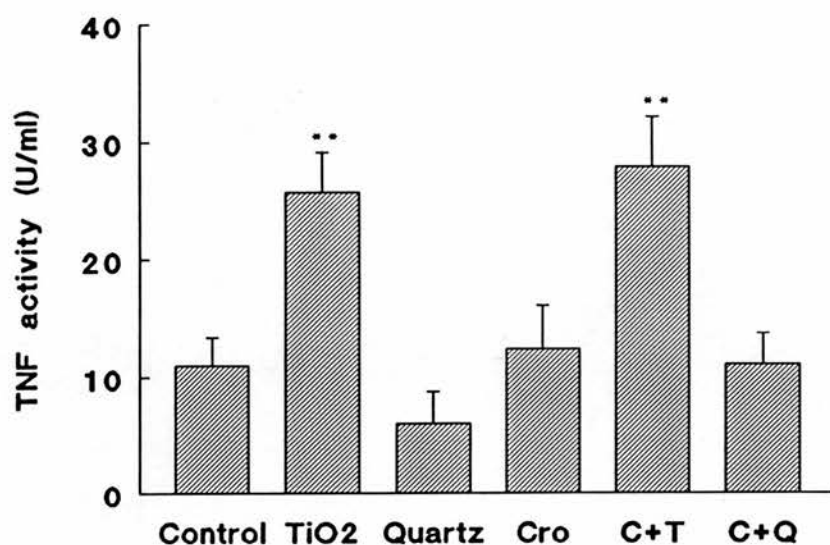


Figure 4.1 TNF production by alveolar leukocytes from rats one month after I/T 2.5 mg dusts

Each bar stands for the mean and SEM of 3 separate experiments performed in triplicate. The dilution of supernatant was 1:64. Asterisk denotes a significant difference from control: **= $P < 0.01$. Cro:-crocidolite asbestos, C+T:-crocidolite plus TiO₂, C+Q:-crocidolite plus quartz.

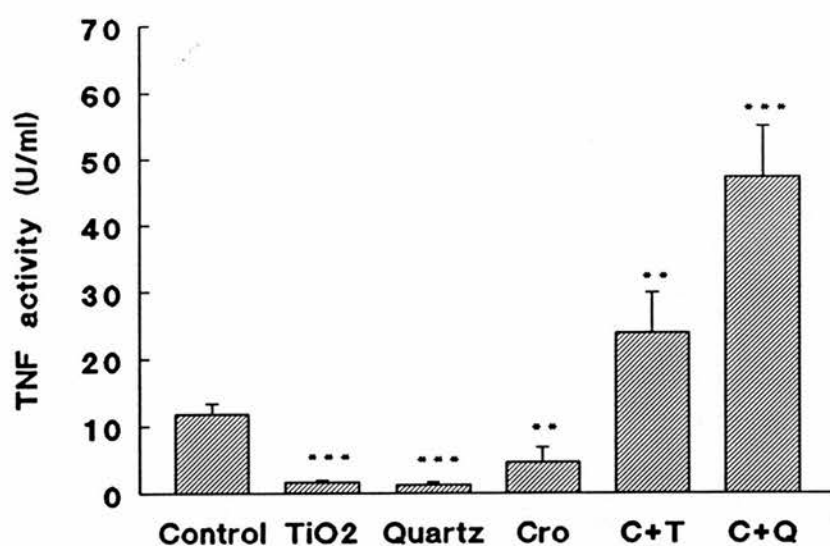


Figure 4.2 TNF production by pleural leukocytes from rats one month after I/T 2.5 mg dusts

Each bar stands for the mean and SEM of 3 separate experiments performed in triplicate wells. The dilution of supernatant was 1:64. Asterisk denotes a significant difference from control: **= $P < 0.01$ and ***= $P < 0.001$. Abbreviations as for Figure 4.1.

Figure 4.3 shows that after all of dusts I/T individually or in combination, bronchoalveolar leukocytes elaborated significantly increased IL-1 activity in culture compared to control. However, no important interaction between those dusts could be observed. Among those dusts, TiO_2 had the greatest stimulating effect, whereas $\text{DQ}_{1,2}$ had the least.

IL-1 production by pleural leukocytes one month after 2.5 mg dusts given by intratracheal instillation is shown on Fig. 4.4. Compared to control, excluding $\text{DQ}_{1,2}$ I/T which stimulated pleural leukocytes to elaborate significantly increased IL-1 activity *in vitro*, other dusts alone or in combination did not significantly alter elaboration of IL-1 by pleural leukocytes.

4.2. *In vitro* studies on the effect of dusts on TNF and IL-1 elaboration by bronchoalveolar macrophages

Normal bronchoalveolar leukocytes, mostly macrophages (>98%), were treated with 50 $\mu\text{g}/\text{ml}$ of crocidolite, TiO_2 and $\text{DQ}_{1,2}$ individually or in combination in culture. Figure 4.5 shows that there were very similar patterns of TNF and IL-1 production by alveolar macrophages in culture treated with different dusts. Compared to control, all of dusts except TiO_2 stimulated macrophages to produce more TNF or IL-1 in culture. Individually, both the highest levels of TNF and IL-1 were found in the culture of alveolar macrophages treated with $\text{DQ}_{1,2}$. The combination of crocidolite and TiO_2 appeared to stimulate the macrophages to produce both TNF and IL-1 additively. However, the combination of crocidolite and $\text{DQ}_{1,2}$ had no stimulating effect over and above their effect individually.

5. Studies on separate fractions of normal pleural leukocytes

As mentioned in Section 2, by using separation techniques, two subpopulations were obtained: a macrophage-enriched population (93% macrophages) and a mast cell-enriched population (76% mast cells and 20% macrophages) (Table 2.1).

5.1. Fibrinolytic activities in separate fractions

The present studies revealed that macrophages are the major source of PAI release in culture (Fig. 2.11). However, pleural leukocytes contain high PA activity intracellularly (Fig. 2.12) and the activity located mainly inside mast cells.

5.2. IL-1 activity in supernatants of separate pleural leukocytes

Figure 5.1 shows that compared to unseparated pleural leukocytes, both macrophage- and mast cell-enriched pleural leukocyte subpopulations produced less IL-1 activity in culture. Moreover, IL-1 activity in supernatant of the mast cell-enriched population was markedly higher than in supernatant of macrophage

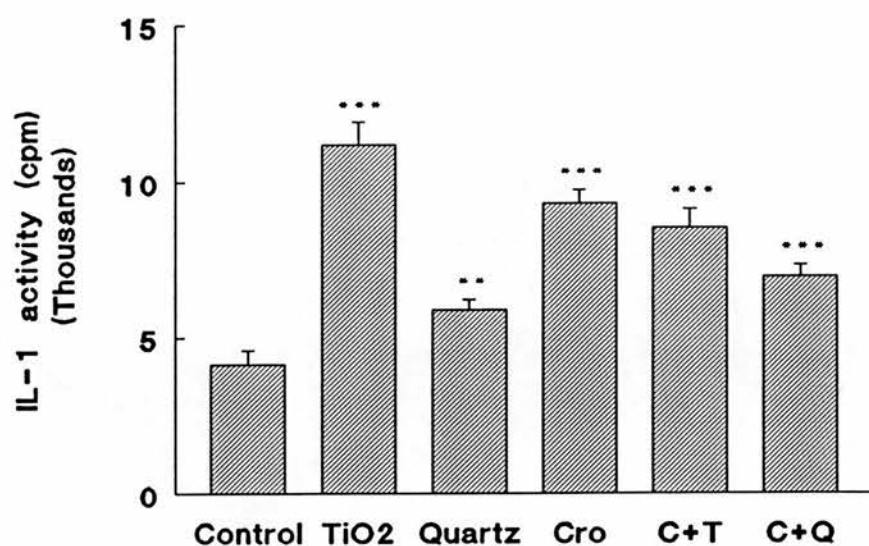


Figure 4.3 IL-1 release by alveolar leukocytes from rats one month after I/T 2.5 mg dusts

Each bar stands for the mean and SEM of 3 separate experiments performed in triplicate wells. The dilution of supernatants was 1:8. Asterisk denotes a significant difference from control: ***= $P < 0.001$. Abbreviations as for Figure 4.1.

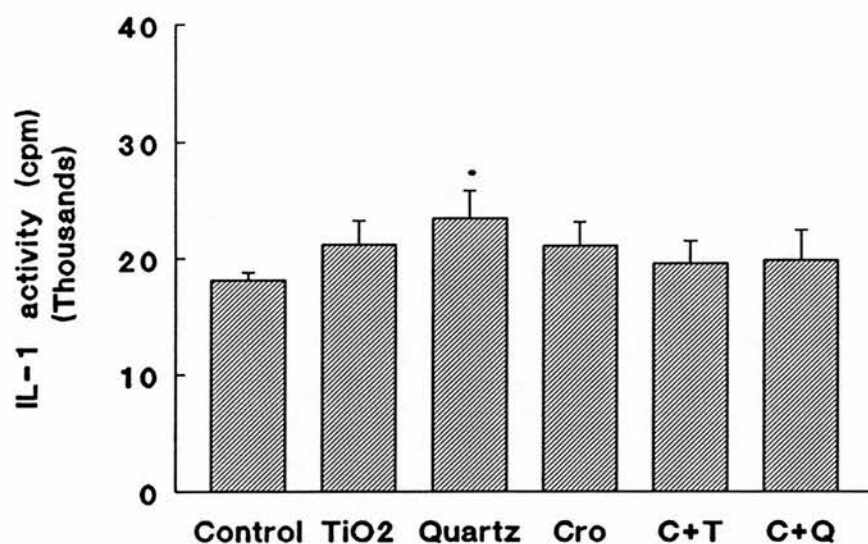


Figure 4.4 IL-1 elaboration by pleural leukocytes from rats one month after I/T 2.5 mg dusts

Each bar stands for the mean and SEM of 3 separate experiments performed in triplicate wells. The dilution of supernatant was 1:8. Asterisk denotes a significant difference from control: *= $P < 0.05$. Abbreviations as for Figure 4.1.

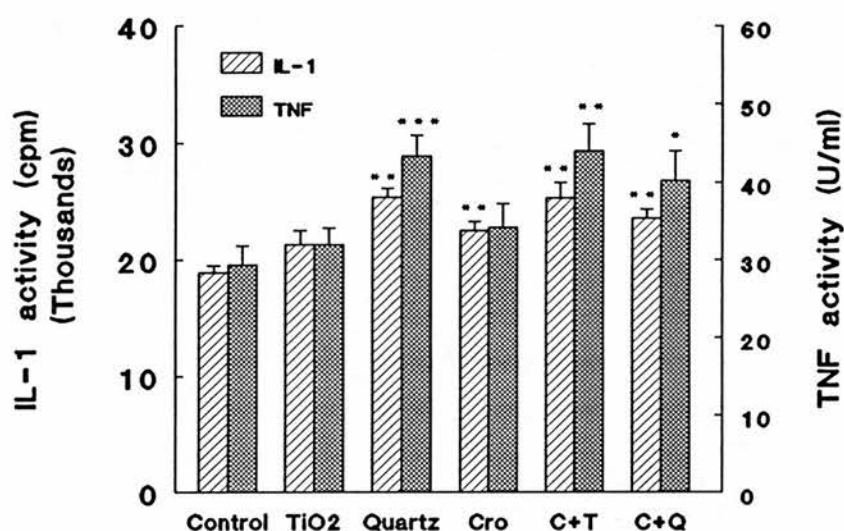


Figure 4.5 TNF and IL-1 production by alveolar macrophages treated with 50 $\mu\text{g/ml}$ dusts *in vitro*

Each bar stands for the mean and SEM of 2-4 separate experiments performed in triplicate wells. The dilution of supernatant was 1:64 for TNF, and 1:16 for IL-1. Asterisk denotes a significant difference from control: $\ast=P<0.05$, $\ast\ast=P<0.01$ and $\ast\ast\ast=P<0.001$. Abbreviations as for Figure 4.1.

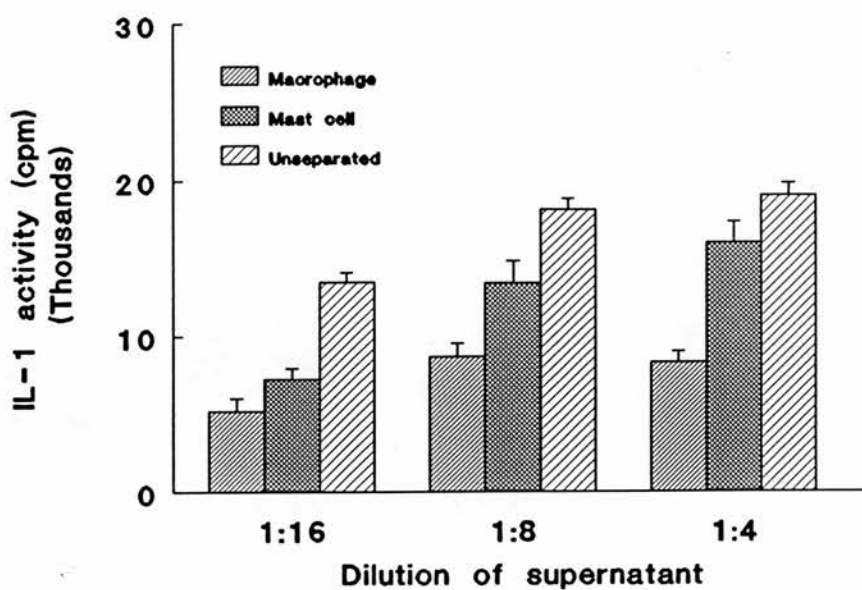


Figure 5.1 IL-1 production by different fractions of normal pleural leukocytes

Each bar represents the mean and SEM of 4-8 separate experiments in triplicate wells.

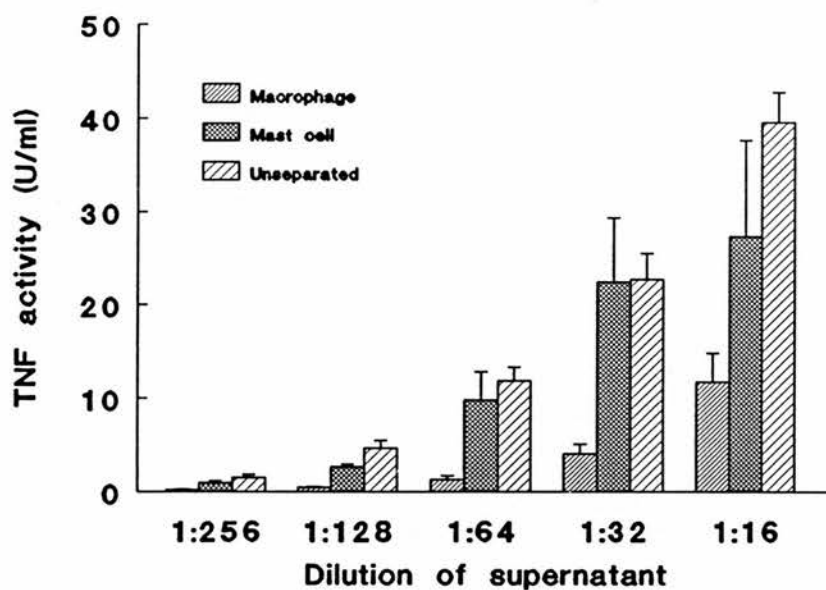


Figure 5.2 TNF production by different fractions of normal pleural leukocytes

Each bar stands for the mean and SEM of 4-8 separate experiments in triplicate wells.

population.

5.3. TNF production by separate pleural leukocytes in culture

A similar pattern to IL-1 was found in TNF production by unseparated and separated pleural leukocytes, i.e., unseparated pleural leukocytes produced more TNF than both separated subpopulation. Between the two separated subpopulations, the mast cell-enriched population produced considerably more TNF in culture than the macrophage-enriched population (Fig. 5.2).

6. Effect of dusts on pleural leukocytes *in vitro* and chemotactic activity in pleural leukocyte supernatant

6.1. TNF and IL-1 elaboration by normal pleural leukocytes treated with 50 μ g/ml dusts *in vitro*

Although we found no evidence to show that pleural leukocytes *in situ* could make contact with intratracheally instilled or inhaled dusts, studies were undertaken to find out the effect of dusts on cytokine production by pleural leukocytes in culture. The results confirmed that there was no significant difference in TNF and IL-1 (Fig. 6.1) production by pleural leukocytes treated with TiO_2 , $\text{DQ}_{1,2}$ or crocidolite asbestos *in vitro*, although dust stimulated increased production of cytokines by the leukocytes compared to control. Quartz did not, however, significantly stimulate TNF secretion although it was increased compared to control.

6.2. Macrophage and PMN chemotaxis caused by pleural leukocyte supernatants

Figure 6.2 shows that there was no chemoattractant for macrophages in control supernatant or in supernatant of pleural leukocytes one day after 5 mg crocidolite I/T, compared to negative control. Although some variation was seen, PMN chemotaxis induced by negative control or by pleural leukocyte supernatants were not significantly different.

7. Mesothelial cell lysis and detachment caused by pleural leukocytes after asbestos I/T

7.1. Pleural leukocyte components after crocidolite asbestos I/T

HAN rats were used in most studies on mesothelial cell injury except the experiments indicated using PVG rats. Fig. 7.1 shows pleural leukocyte components after asbestos I/T. Macrophage numbers increased 14 days after 5 mg crocidolite I/T and reached the highest level at day 30 after instillation. Eosinophil numbers increased from 3 days to 14 days after instillation and then recovered by day 30. The numbers of mast cells increased significantly by day 14 and quickly recovered to normal levels. No PMN could be observed in the pleural leukocytes during the whole experimental period.

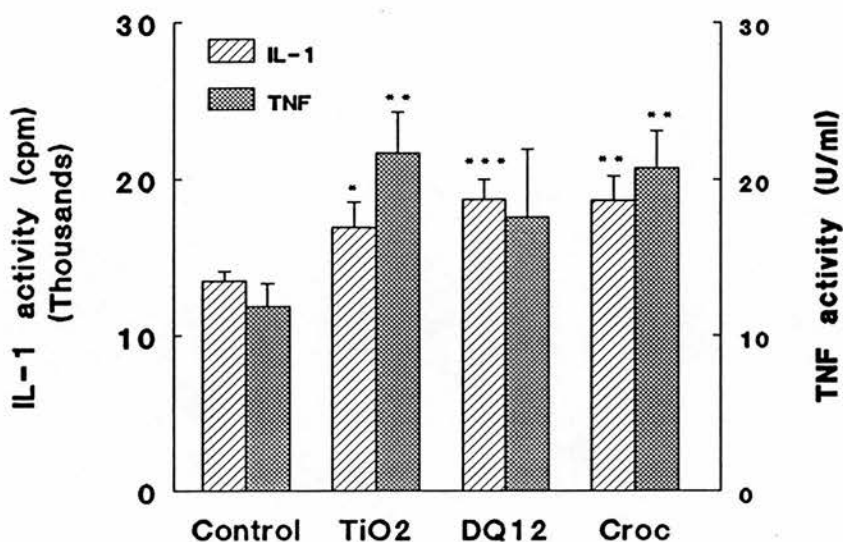


Figure 6.1 TNF and IL-1 production by normal pleural leukocytes treated with 50 µg/ml dusts *in vitro*

Each bar stands for the mean and SEM of 2 separate experiments in triplicate wells. The dilution of supernatant was 1:64 for TNF and 1:16 for IL-1. Asterisk denotes a significant difference from control: *=P<0.05, **=P<0.01 and ***=P<0.001.

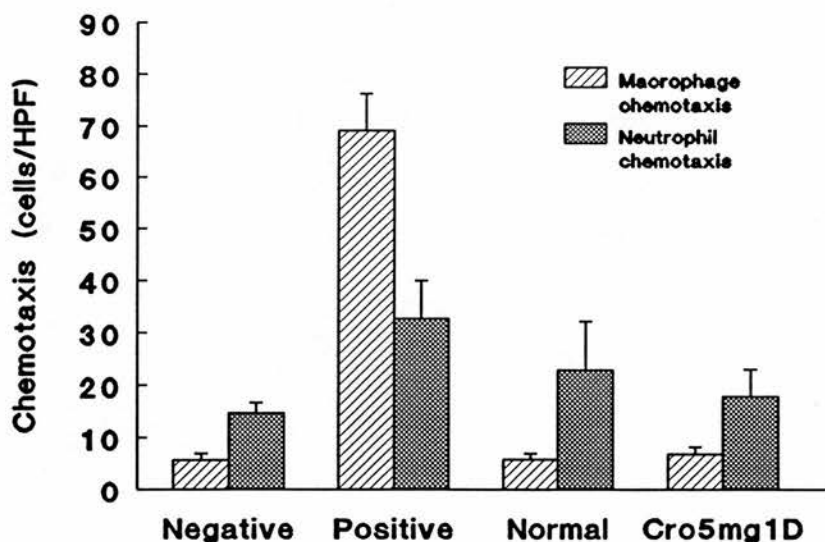


Figure 6.2 Macrophage and neutrophil chemotaxis caused by pleural leukocyte supernatants

Each bar stands for the mean and SEM of 2-4 separate experiments performed in duplicate.

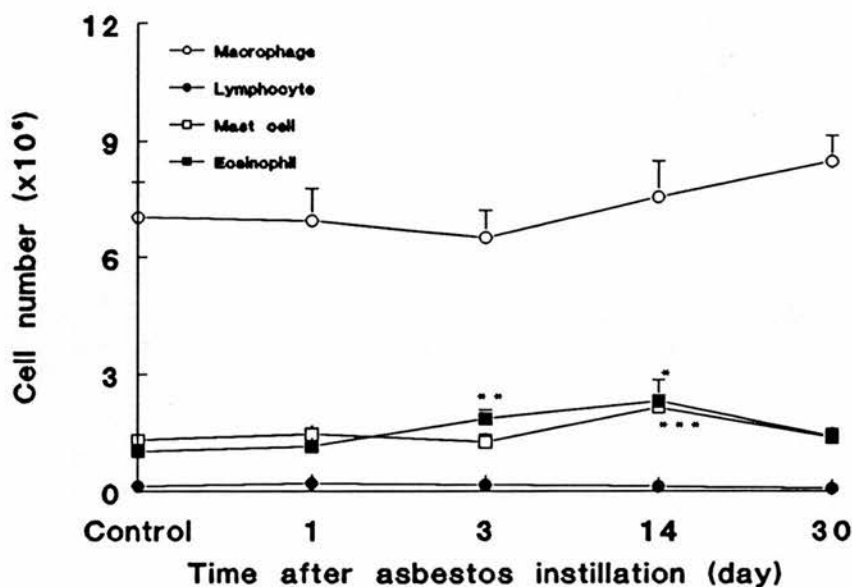


Figure 7.1 HAN rat pleural leukocyte components at various times after I/T 5 mg crocidolite asbestos

Each point represents the mean and SEM of 7-12 separate experiments. Asterisk denotes a significant difference from control: $*=P<0.05$, $**=P<0.01$ and $***=P<0.001$.

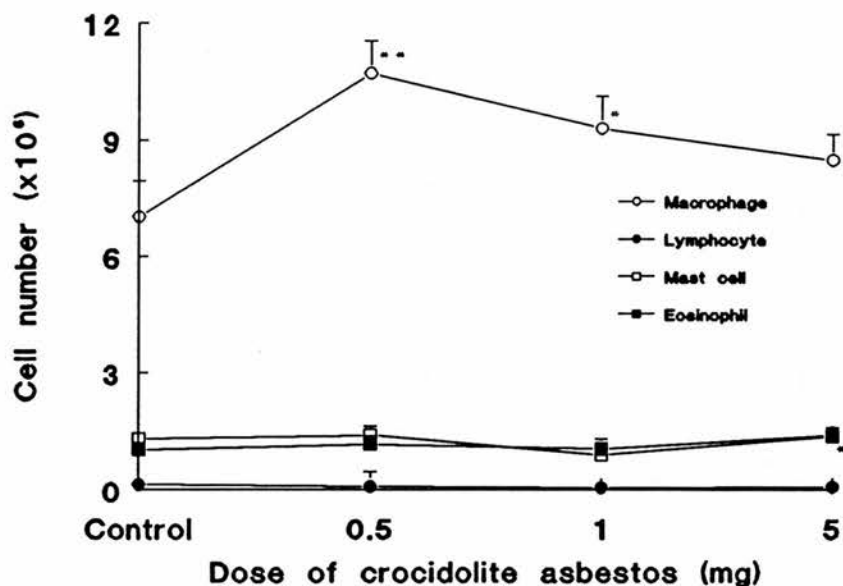


Figure 7.2 HAN rat pleural leukocyte components one month after I/T different doses of crocidolite

Each point represents the mean and SEM of 6-12 separate experiments. Asterisk denotes a significant difference from control: $*=P<0.05$, $**=P<0.01$.

Figure 7.2 illustrates that one month after various doses of crocidolite asbestos I/T, there was an increase of macrophages in the pleural leukocyte population. However, it seemed that lower doses of asbestos caused more recruitment of macrophages. Other cell components of pleural leukocyte had no marked changes though the instilled doses of asbestos increased from 0.5 mg to 5 mg.

7.2. Mesothelial cell lysis and detachment caused by pleural leukocytes after crocidolite asbestos I/T

Figure 7.3 shows that there was no effects on mesothelial cell lysis and detachment caused by increasing numbers of normal pleural leukocytes co-cultured with the mesothelial cells.

However, one day after 5 mg crocidolite I/T, the pleural leukocytes caused remarkable mesothelial cell detachment which increased in response to augmentation of the numbers of pleural leukocytes (Fig. 7.4-1), lysis of mesothelial cells was not influenced.

By three days after 5 mg crocidolite asbestos I/T, although pleural leukocytes still caused slight detachment of mesothelial cells in culture at higher ratios of pleural leukocytes to mesothelial cells, this was much less than that resulted from pleural leukocytes at one day (Fig.7.4-2). There was no detectable mesothelial cell lysis.

From 14 days to 30 days after asbestos I/T, pleural leukocytes did not cause any measurable mesothelial cell lysis or detachment (Figs. 7.4-3, 7.4-4).

Nevertheless, one month after intratracheal instillation of asbestos, although the doses of crocidolite asbestos varied from 0.5, 1 to 5 mg, no mesothelial cell detachment and lysis could be detected (Table 7.1).

7.3. Mesothelial cell lysis and detachment caused by proteases

To reveal the mechanism of mesothelial cell detachment caused by pleural leukocytes, the possible role of proteases, i.e., trypsin, elastase and collagenase, were evaluated. Figure 7.5 shows that elastase and collagenase did not cause important changes of mesothelial cell lysis over the whole dose range. Trypsin induced slight increase of mesothelial cell lysis at higher doses. However, trypsin and elastase in culture resulted in apparent increase of mesothelial cell detachment with the concentrations being higher than 10 $\mu\text{g/ml}$ (Fig. 7.5). There was no any detectable mesothelial cell detachment injury caused by collagenase.

8. The activities of normal pleural lavage fluids

8.1. Fibrinolytic activity of pleural lavage fluid

Pleural lavage fluid had neither PA nor PAI activity (Fig. 2.12).

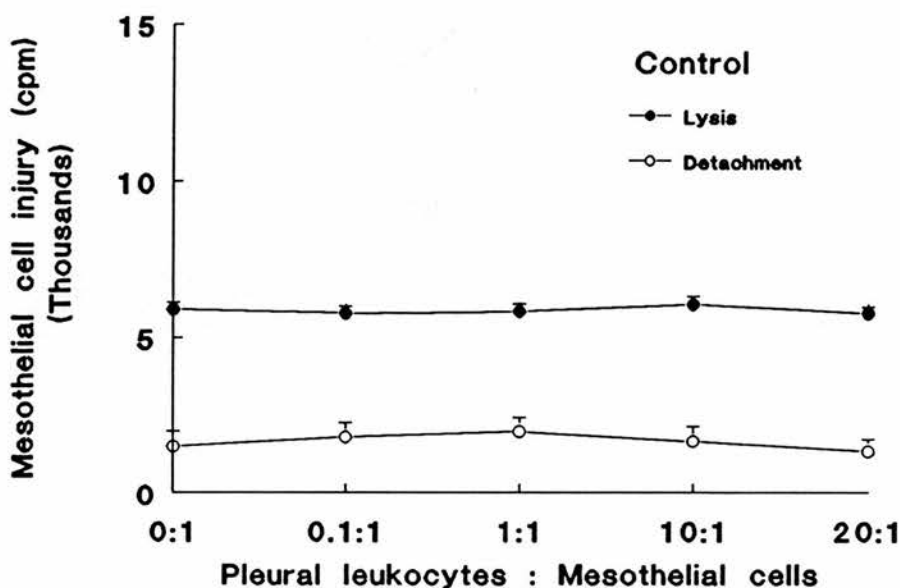


Figure 7.3 Mesothelial cell lysis and detachment injury caused by control pleural leukocytes

Each point stands for the mean and SEM of 11 separate experiments in triplicate.

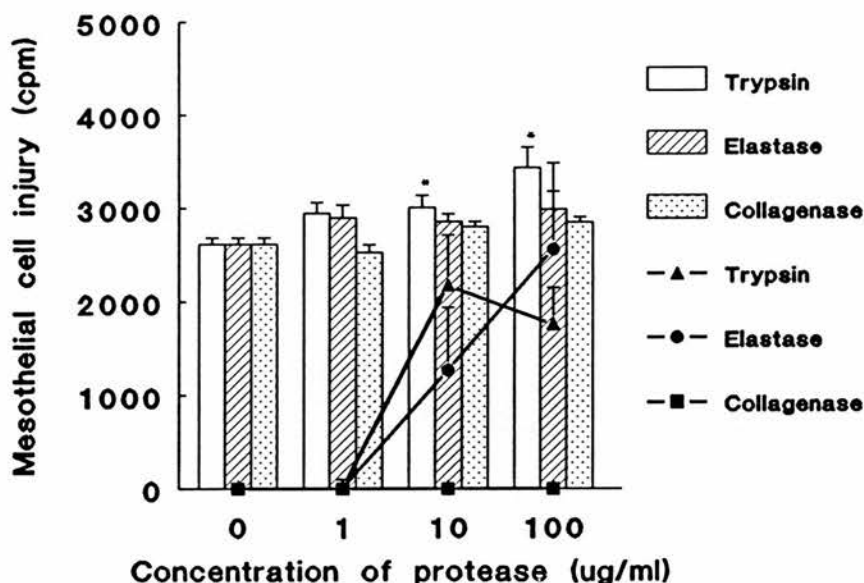


Figure 7.5 Mesothelial cell lysis and detachment caused by proteases

Each bar represents the mean and SEM of 2 separate experiments performed in triplicate. Bar represents mesothelial cell lysis and line represents mesothelial cell detachment.

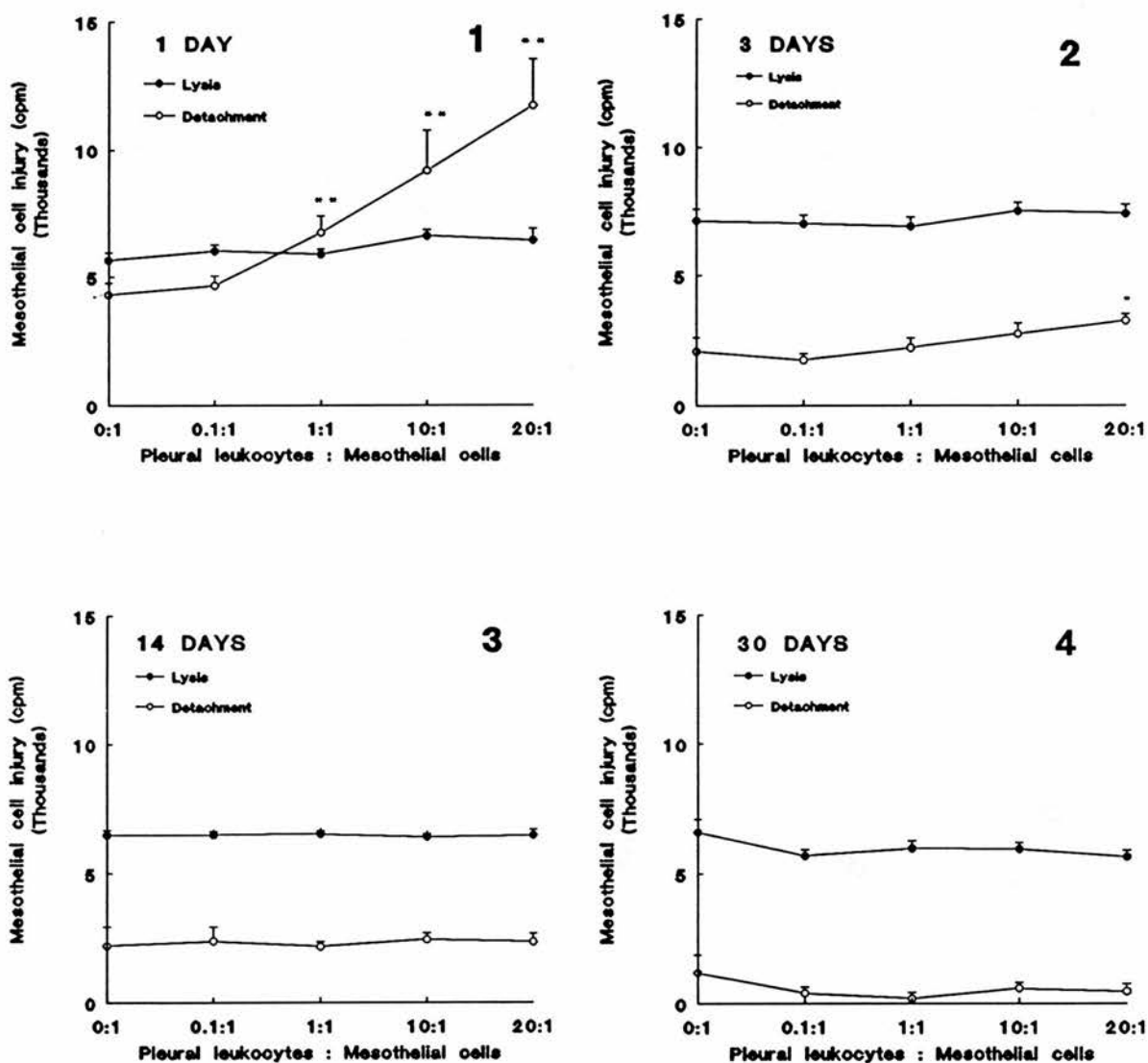


Figure 7.4 (1-4) Mesothelial cell lysis and detachment injury caused by pleural leukocytes from rats at various times after I/T 5 mg crocidolite

Each point represents mean and SEM of 3-9 separated experiments in triplicate. Compared to the wells without the addition of pleural leukocytes, * means $P < 0.05$ and ** $P < 0.01$.

Table 7.1 CELL LYSIS AND DETACHMENT CAUSED BY PLEURAL LEUKOCYTES FROM RATS ONE MONTH AFTER VARIOUS DOSES OF CROCIDOLITE I/T

Dose (mg)	Ratio of pleural leukocytes : mesothelial cells				
	0:1	0.1:1	1:1	10:1	20:1
<u>0.5 mg</u>					
Lysis	3938(1063) ^a	3785(701)	3737(722)	3835(890)	3721(918)
Detach	0	0	0	0	0
<u>1 mg</u>					
Lysis	3644(479) ^b	3674(561)	3733(490)	3857(518)	3887(595)
Detach	0	0	0	0	0
<u>5 mg</u>					
Lysis	6605(1521) ^c	5702(1123)	5977(1536)	5951(1310)	5658(1284)
Detach	1190(2045)	401(1240)	195(1187)	587(1117)	473(1501)

^an=6 separate experiments, ^bn=6 separate experiments and ^cn=9 separate experiments in triplicate. Detach:-detachment. Results are presented as mean(SD) cpm.

8.2. IL-1 and TNF activities in pleural lavage fluid

There was no important IL-1 (Table 8.1) or TNF (Table 8.2) activities in normal pleural lavage fluid. The detected activity was the reflection of background of the assays.

Table 8.1 IL-1 ACTIVITY IN NORMAL PLEURAL LAVAGE FLUID AND PLEURAL LEUKOCYTE SUPERNATANT

Sample	Dilution		
	1:16	1:8	1:4
Pleural lavage fluid	343 (144) ^a	255 (92)	377 (212)
Pleural leukocyte supernatant	13486 (4161) ^b	18146 (4573)	19056 (4975)

^an= 4, ^bn= 15 separate experiments in triplicate respectively. Results are presented as mean(SD) cpm.

Table 8.2 TNF ACTIVITY IN NORMAL PLEURAL LAVAGE FLUID AND PLEURAL LEUKOCYTE SUPERNATANT

Sample	Dilution		
	1:64	1:32	1:16
Pleural lavage fluid	0.35(0.49) ^a	0.25(0.34)	0.23(0.35)
Pleural leukocyte supernatant	11.87(6.85) ^b	22.73(11.1)	39.52(15.16)

^a_n= 3, ^b_n=7 separate experiments in triplicate. Results are presented as mean(SD) U/ml.

8.3. Pleural lavage fluid inhibits IL-1 and TNF activities in pleural leukocyte supernatants

Normal pleural lavage fluid inhibited both IL-1 activity (Fig. 8.1) and TNF activity (Fig. 8.2) in normal pleural leukocyte supernatants in a lavage fluid concentration-related manner.

8.4. Pleural lavage fluid inhibits the activities of the IL-1 and TNF standards

After the addition of pleural lavage fluid to IL-1 β standard in the C3H mouse thymocyte assay, the activity of IL-1 standard in stimulating the thymocyte growth was inhibited in a lavage fluid concentration-related manner (Fig. 8.3).

Figure 8.4 exhibits the inhibitory effect of pleural lavage fluid on TNF α standard. There was again, a dose response of pleural lavage fluid with respect to its inhibitory effect on TNF α .

8.5. Pleural lavage fluid stimulates mesothelial cell proliferation

Figure 8.5 shows that pleural lavage fluid from rats within 14 days after 5 mg crocidolite asbestos I/T had no detectable stimulating effect on mesothelial cell proliferation compared to control. However, when it was one month after crocidolite I/T, pleural lavage fluid stimulated the proliferation of mesothelial cells in culture.

9. The activities of pleural leukocyte lysate, mesothelial cell supernatant and lysate

9.1. Fibrinolytic activity of pleural leukocyte lysates

Studies with pleural leukocyte lysates illustrated that the pleural leukocyte lysate contained high plasminogen activator activity (Fig. 2.9).

9.2. TNF and IL-1 activities of pleural leukocyte lysates

Table 9.1 illustrates that there was no detectable TNF activity in pleural

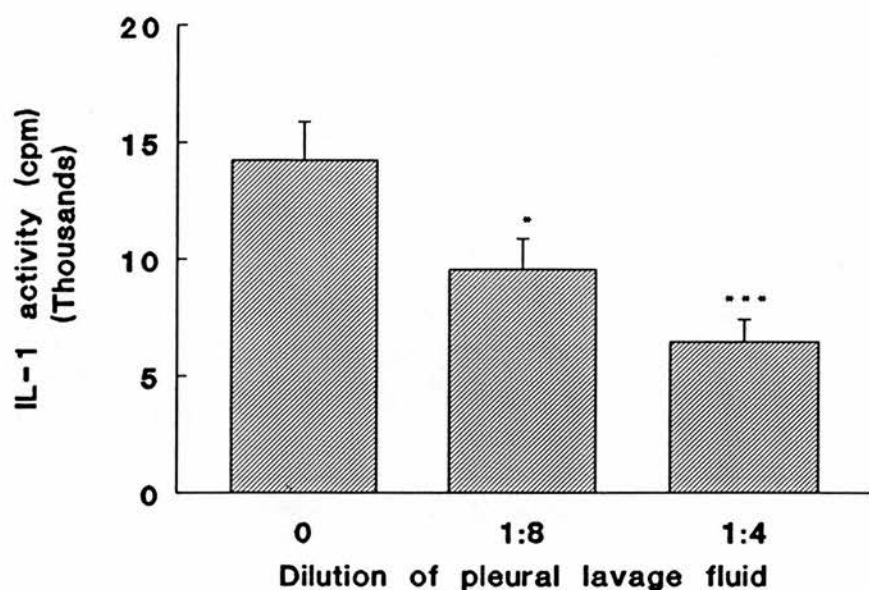


Figure 8.1 Inhibition of IL-1 activity in pleural leukocyte supernatant by pleural lavage fluid

Each bar stands for the mean and SEM of 12 separate experiments carried out in triplicate. Asterisk denotes a significant difference from control: $*=P<0.05$, $***=P<0.001$.

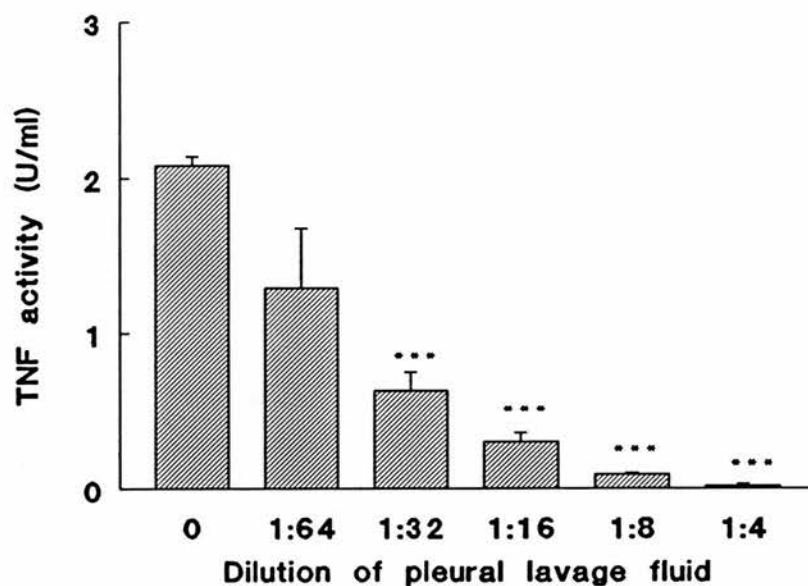


Figure 8.2 Inhibition of TNF activity in pleural leukocyte supernatant by pleural lavage fluid

Each bar stands for the mean and SEM of 4 separate experiments carried out in triplicate. Asterisk denotes a significant difference from control: $***=P<0.001$.

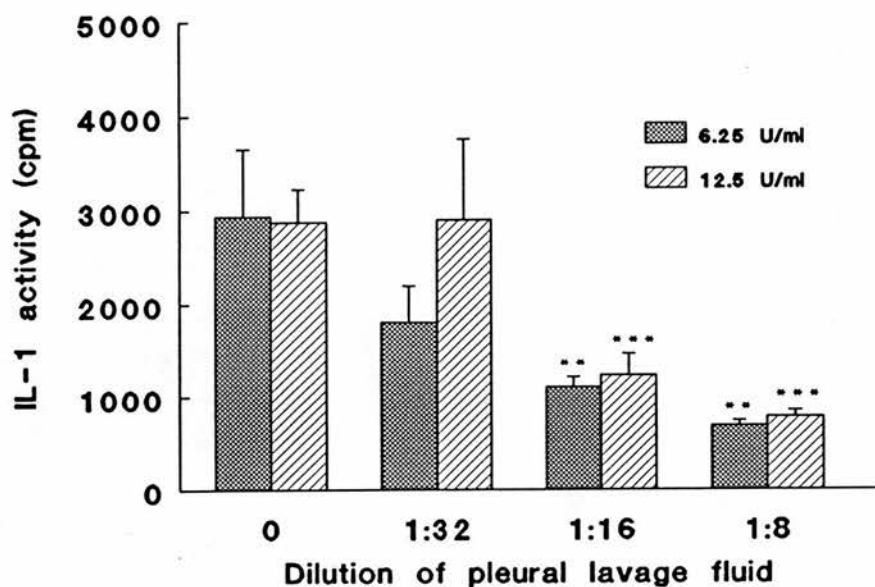


Figure 8.3 Normal pleural lavage fluid inhibits the activity of the IL-1 β standard

Each bar stands for the mean and SEM of 2-9 separate experiments carried out in triplicate. Asterisk denotes a significant difference from corresponding IL-1 activity of control: ** = P < 0.01 and *** = P < 0.001.

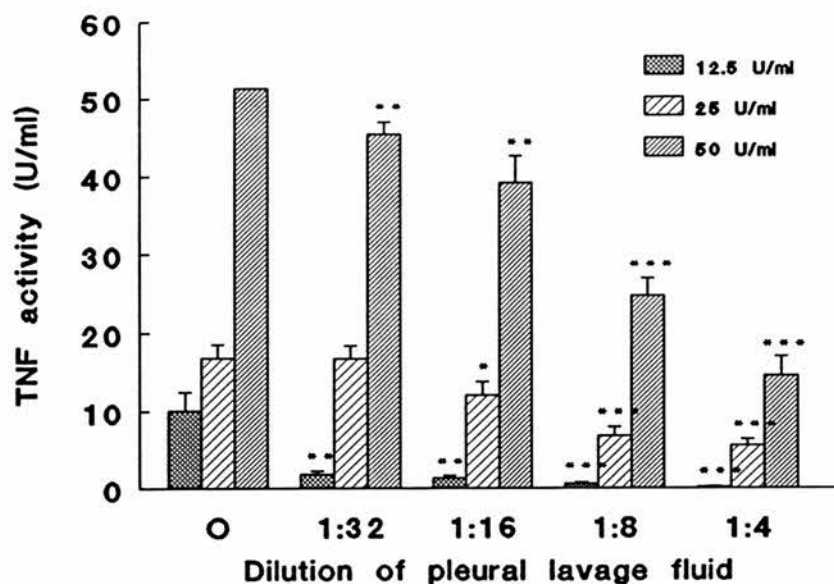


Figure 8.4 Normal pleural lavage fluid inhibits the activity of the TNF α standard

Each bar stands for the mean and SEM of 4-8 separate experiments carried out in triplicate. Asterisk denotes a significant difference from corresponding TNF activity of control: * = P < 0.05, ** = P < 0.01 and *** = P < 0.001.

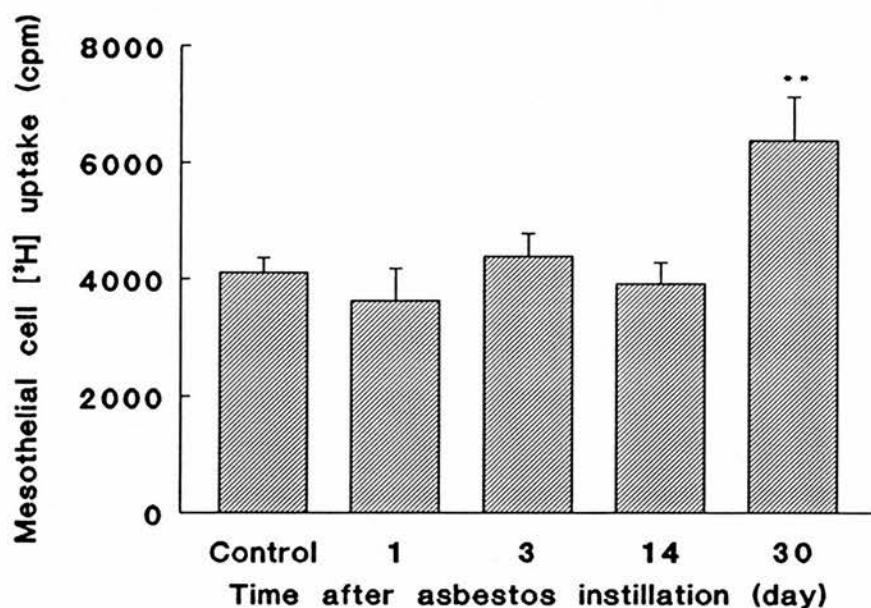


Figure 8.5 Mesothelial cell proliferation caused by pleural lavage fluid from rats at various times after I/T 5 mg crocidolite asbestos

Each bar stands for the mean and SEM of 8-17 separate experiments in triplicate. The dilution of pleural lavage fluid was 1:4. Compared to control, ** means $P < 0.01$.

leukocyte lysates. In addition, no inhibitory effect of pleural leukocyte lysate on the $\text{TNF}\alpha$ standard (Table 9.2). Table 9.3 shows no IL-1 activity in the pleural leukocyte lysate.

Table 9.1 TNF ACTIVITY IN THE PLEURAL LEUKOCYTE LYSATES

PLL ^a (Dilution)	1:64	1:32	1:16	1:8	1:4
TNF(U/ml) ^b	0.02(0.02)	0.02(0.02)	0.02(0.01)	0.02(0.02)	0.02(0.02)

^aPLL: Pleural leukocyte lysate; ^bTNF activity detected by L929 cell line assay; results are presented as mean(SD) of 6 separate experiments in triplicate.

Table 9.2 THE EFFECT OF PLEURAL LEUKOCYTE LYSATE ON $\text{TNF}\alpha$ STANDARD

PLL ^a (Dilution)	0	1:64	1:32	1:16	1:8	1:4
TNF(U/ml) ^b	2.3(0)	1.9(1.2)	2.3(1.5)	2.1(1.0)	1.9(1.4)	1.7(1.8)

^aPLL: Pleural leukocyte lysate; ^bTNF activity after the addition of pleural leukocyte lysate into the culture of $\text{TNF}\alpha$ standard; results are presented as mean(SD) of 3 separate experiments in triplicate.

Table 9.3 IL-1 ACTIVITY IN THE PLEURAL LEUKOCYTE LYSATES

PLL ^a (Dilution)	1:16	1:8	1:4
IL-1 activity (cpm) ^b	566 (172)	707 (185)	493 (186)

^aPLL: Pleural leukocyte lysate; ^bIL-1 activity detected by C3H mouse thymocyte proliferation assay; and results are presented as mean(SD) of 4 separate experiments in triplicate.

9.3. TNF and IL-1 activities of mesothelial cell supernatants

Table 9.4 indicates no detectable TNF activity in the supernatants of rat mesothelial cells. In addition, no detectable IL-1 activity was present in the supernatants of rat mesothelial cells (Table 9.5). There was also no important inhibitory effect of mesothelial cell supernatants on $\text{TNF}\alpha$ standard (Table 9.6).

Table 9.4 TNF ACTIVITY IN MESOTHELIAL CELL SUPERNATANTS

MCS ^a (Dilution)	1:64	1:32	1:16	1:8	1:4
TNF(U/ml) ^b	0.30(0.31)	0.17(0.14)	0.15(0.13)	0.20(0.16)	0.13(0.14)

^aMCS: Mesothelial cell supernatants; ^bTNF activity detected by L929 cell line assay; n=3 separate experiments in triplicate, results are shown as mean(SD).

Table 9.5 IL-1 ACTIVITY IN MESOTHELIAL CELL SUPERNATANTS

MCS ^a (Dilution)	1:16	1:8	1:4
IL-1 activity (cpm) ^b	281 (152)	271 (80)	323 (73)

^aMCS: Mesothelial cell supernatants; ^bIL-1 activity detected by C3H mouse thymocyte proliferation assay; and results are presented as mean(SD) of 2 separate experiments in triplicate.

Table 9.6 THE EFFECT OF MESOTHELIAL CELL SUPERNATANTS ON TNF α STANDARD

MCS ^a (Dilution)	0	1:64	1:32	1:16	1:8	1:4
TNF (U/ml) ^b	46.4(6.9)	40.1(2)	41.5(0)	41.5(0)	43.1(2.2)	44.6(0)

^aMCS: Mesothelial cell supernatants; ^bTNF activity after the addition of the supernatants into the culture of TNF α standard; results are presented as mean(SD) of 2 separate experiments in triplicate.

9.4. TNF and IL-1 activities of mesothelial cell lysates

Tables 9.7 and 9.8 show that there were no detectable TNF and IL-1 activities in the lysates of rat mesothelial cells.

Table 9.7 TNF ACTIVITY IN MESOTHELIAL CELL LYSATES

MCL ^a (Dilution)	1:64	1:32	1:16	1:8	1:4
TNF(U/ml) ^b	0.14(0.19)	0.08(0.07)	0.08(0.06)	0.07(0.06)	0.05(0.05)

^aMCL: Mesothelial cell lysates; ^bTNF activity detected by L929 cell line assay; and results are presented as mean(SD) of 4 separate experiments in triplicate.

Table 9.8 IL-1 ACTIVITY IN MESOTHELIAL CELL LYSATES

MCL ^a (Dilution)	1:16	1:8	1:4
IL-1 activity (cpm) ^b	395 (194)	307 (52)	493 (669)

^aMCL: Mesothelial cell lysates; ^bIL-1 activity detected by C3H mouse thymocyte proliferation assay; and results are presented as mean(SD) of 3 separate experiments in triplicate.

10. Interleukin-2 (IL-2) activity in pleural leukocyte supernatants

To demonstrate that the observed IL-1 activity was not due to IL-2 released from contaminating lymphocytes (Yamaguchi *et al*, 1989), the pleural leukocyte supernatants were tested for IL-2 activity using the CTLL cell line which proliferates specifically ^{in response} to IL-2. Figure 10.1 illustrates that there was no important IL-2 activity in the supernatants of control pleural leukocytes and supernatants of the leukocytes at various times (from 1 day to 30 days) after crocidolite asbestos I/T at various doses (from 5 mg to 10 mg), whilst the positive control, IL-2 standard, gave a marked response. IL-2 activities were also not detectable in the supernatant of pleural leukocytes from rats various times after *C.parvum* I/T (Fig. 10.1).

11. Validation of assays

11.1. TNF L929 cell line assay

The effect of IL-1 β standard on TNF L929 cell line assay was shown in Table 11.1. It was apparent that up to 12.5 U/ml, IL-1 standard sample had no effect on L929 cell line assay.

Table 11.1 THE EFFECT OF IL-1 β STANDARD ON L929 CELL LINE ASSAY

IL-1 β (U/ml)	0.63	1.56	3.13	6.25	12.5
TNF(U/ml)	0.08(0.03) ^a	0.06(0.03)	0.06(0.03)	0.04(0.03)	0.04(0.03)

^aTNF activity detected by L929 cell line assay, and the results are presented as mean(SD) of 3 separate experiments in triplicate.

Table 11.2 indicates that F10 medium plus 2% BSA had no effect on L929 cell line assay.

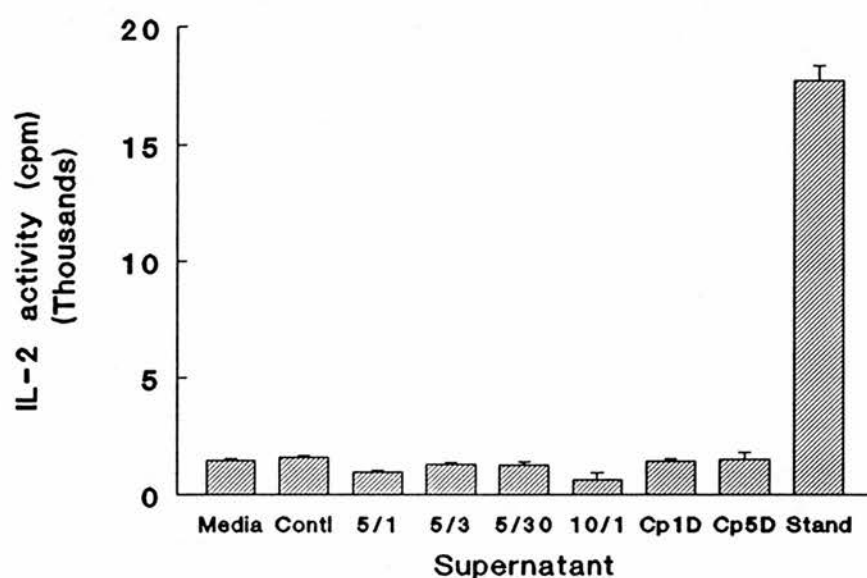


Figure 10.1 IL-2 activity in pleural leukocyte supernatants after I/T crocidolite asbestos or *C.parvum*

Each bar represents the mean and SEM of 2-12 separate experiments. Abbreviations represent: Media:- medium alone control; Contl:- control supernatant; 5/1:- 1 day after I/T 5mg crocidolite; 5/3:- 3 day after I/T 5mg crocidolite; 5/30:- 30 days after I/T 5mg crocidolite; 10/1:- 1day after I/T 10 mg crocidolite; Cp1D:- 1 day after I/T 1.4 mg *C.parvum*; Cp5D:- 5 days after I/T 1.4 mg *C.parvum*; Stand:- IL-2 standard. The dilution of all of pleural leukocyte supernatants was 1:2 and the dose of IL-2 standard was 10 U/ml.

Table 11.2 THE EFFECT OF F10 MEDIUM PLUS 2% BSA ON L929 CELL LINE ASSAY

F10+2%BSA (Dilution)	1:32	1:16	1:8	1:4
TNF (U/ml)	0.05(0.06) ^a	0.03(0.02)	0.02(0.01)	0.02(0.01)

^aTNF activity detected by L929 cell line assay and the results are presented as mean(SD) of 3 separate experiments in triplicate.

11.2. IL-1 C3H mouse thymocyte proliferation assay

The effect of TNF α on C3H mouse thymocyte proliferation assay is shown in Table 11.3. There was negligible effect of TNF α in the assay.

Table 11.3 THE EFFECT OF TNF α ON C3H MOUSE THYMOCYTE PROLIFERATION ASSAY

TNF α (U/ml)	25	50	100
IL-1 activity (cpm)	819 (292) ^a	771 (397)	1003 (664)

^aIL-1 activity (cpm) detected by C3H mouse thymocyte proliferation assay and the results are presented as mean(SD) of 3 separate experiments in triplicate.

11.3. Fibrinolysis assay

TNF α up to 1000 U/ml was added into fibrinolysis assay. Results indicated that TNF α did not interfere with PAI activity in pleural leukocyte supernatants (Table 11.4).

Table 11.4 EFFECT OF TNF α ON FIBRINOLYSIS INHIBITION CAUSED BY PLEURAL LEUKOCYTE SUPERNATANT

TNF (U/ml)	0	62.5	125	250	500	1000
% inhibition	48.86	40.97	47.01	42.56	38.48	46.08
	(17.14)	(7.25)	(13.64)	(10.13)	(5.13)	(6.63)

Results are presented as mean(SD) of 6 separate experiments in triplicate. Fibrinolysis was induced by urokinase and PLG. The dilution of supernatant of pleural leukocytes was 1:16.

12. Reactive pleural inflammation caused by intratracheal instillation of *C.parvum*

12.1. Alveolar and pleural leukocyte populations after I/T *C.parvum*

Normal rat alveolar leukocyte populations contained more than 95% macrophages with a total cell number of less than 5×10^6 . One day after

intratracheal instillation of *C.parvum*, large number of PMN were recruited to the bronchoalveolar space and this increased the total cell number to 27×10^6 , although macrophage number remained at the same levels (Fig 12.1). By 3 days after instillation, numbers of PMN had decreased dramatically and were minimal by day 5. However, the numbers of macrophages were markedly increased by day 3 and remained elevated at day 5.

Figure 12.2 shows that six hours after *C.parvum* I/T, except for some variation in numbers of mast cells and eosinophils, changes of other cell components in the pleural space were negligible. However, one day after instillation there was a slight increase in numbers of macrophages, but a marked increase in numbers of PMNs in the pleural space. The peak of PMNs at day 1 disappeared quickly and no PMNs were found by day 5. However, numbers of macrophages increased dramatically from day 3 and remained high at day 5. When the components of pleural leukocytes were compared with those of alveolar leukocytes, the patterns of alteration in the macrophage and PMN populations were very similar.

12.2. Analysis of fluorescent polystyrene microspheres (beads) and *C.parvum* organisms in both alveolar and pleural cavity

When beads were intratracheally instilled alone, they induced a moderate alveolitis, which was characterized by 24% PMNs in the bronchoalveolar lavage (Table 12.1). However, in the pleural space, little effect was seen.

Table 12.1 ALVEOLAR AND PLEURAL LEUKOCYTE POPULATIONS AFTER BEADS AND/OR C.PARVUM I/T

Treatment	Total No ($\times 10^6$)	Differential percentage				
		Macrophages	PMN	Lymphocytes	Mastcells	Eosinophils
<u>Alveolar leukocytes</u>						
B alone	7.36(1.26)	75(3.5)	24(2.8)	1(0.7)	0	0
B+Cp(day1)	58.97(2.95)	16(2.1)	84(2.8)	0	0	0
B+Cp(day5)	13.87(1.76)	81(9.5)	18(9.5)	1(0)	0	0
<u>Pleural leukocytes</u>						
B alone	4.10(0.70)	76(6.4)	0	1(0)	16(3.5)	7(2.8)
B+Cp(day1)	11.80(3.38)	60(0.7)	29(11)	0	6(5.7)	5(5.7)
B+Cp(day5)	9.47(0.55)	86(0)	3(1.5)	6(1)	4(1.7)	1(0.6)

Results are presented as mean(SD) of two separate experiments. B: beads; Cp:

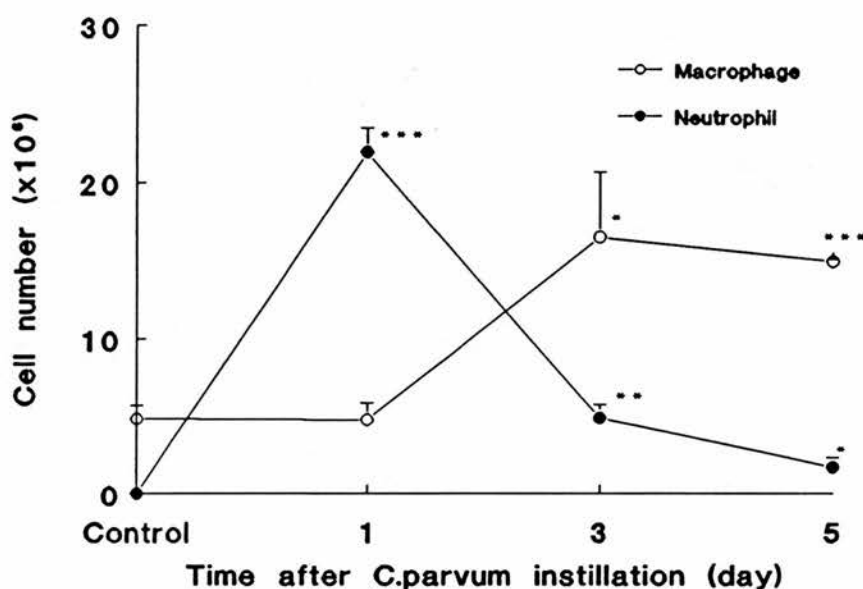


Figure 12.1 Alveolar leukocyte populations at various times after I/T *C. parvum*

Each point shows mean and SEM of 3 separate experiments. Asterisk denotes a significant difference from control: $*=P<0.05$, $**=P<0.01$ and $***=P<0.001$.

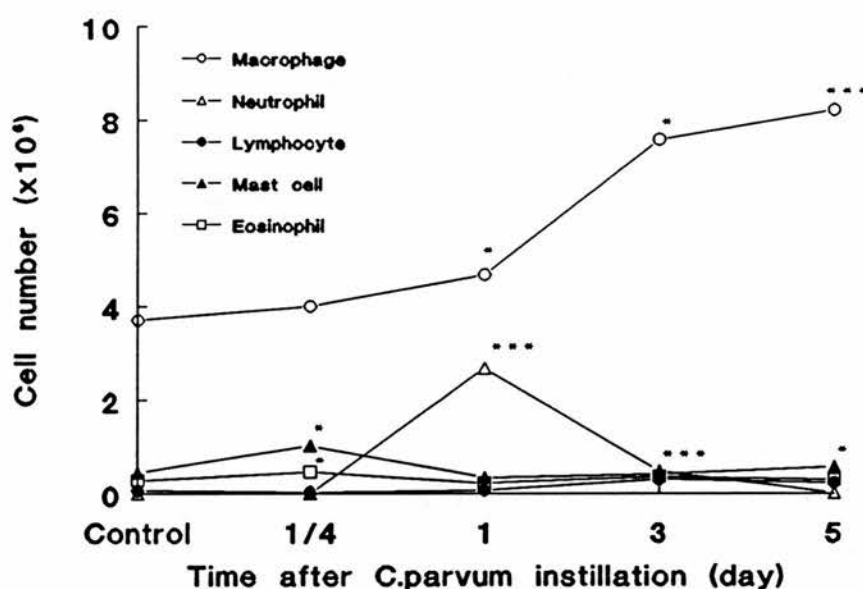


Figure 12.2 Pleural leukocyte populations at various times after I/T *C. parvum*

The points show mean and SEM of 2 to 13 separate experiments. For clarity SEM, which varies from 8% to 30%, is not presented in the graph. Asterisk denotes a significant difference from control: $*=P<0.05$, $***=P<0.001$.

C.parvum.

Counting of beads showed that a high percentage (72%) of alveolar macrophages engulfed beads and most macrophages (>70%) were found to contain more than 10 beads (Table 12.2). This suggested that after instillation of beads, macrophages were very active in removing them from the lung surface. However, beads were negligible in the pleural leukocytes.

Table 12.2 ANALYSIS OF BEADS IN THE ALVEOLAR AND PLEURAL LEUKOCYTES FOLLOWING BEADS AND/OR C.PARVUM I/T

Treatment	% of cells with beads	Distribution of beads in cells (%)		
		1-5	5-10	>11
<u>Alveolar macrophages</u>				
B alone	72 (7.1)	12 (8.5)	17 (4.2)	71 (12.7)
B+Cp (day1)	37 (6.4)	57 (7.1)	26 (8.5)	17 (1.4)
B+Cp (day5)	15 (1.5)	52 (8.7)	25 (6.2)	23 (2.6)
<u>Alveolar PMNs</u>				
B alone	33 (18.4)	90 (2.8)	9 (1.4)	1 (1.4)
B+Cp (day1)	4 (0.7)	100 (0)	0	0
B+Cp (day5)	0 (0)	0	0	0
<u>Pleural macrophages</u>				
B alone	<1	100	0	0
B+Cp (day1)	<1	100	0	0
B+Cp (day5)	0	0	0	0
<u>Pleural PMNs</u>				
B alone	0	0	0	0
B+Cp (day1)	<1	100	0	0
B+Cp (day5)	0	0	0	0

Results are presented as mean(SD) of two separate experiments. Abbreviation is the same as Table 12.1.

One day after *C.parvum* and beads I/T together, a severe inflammatory response was observed in the alveolar space with the total number of alveolar leukocytes being up to 8 fold greater than with bead instillation alone, PMN accounted for more than 80% of the total alveolar population (Table 12.1). Due to the massive recruitment of PMN into the alveolar space or a turnover of macrophages entering and leaving the lung, the percentages of macrophages

containing beads decreased from 72% to 37% and the distribution of beads in the macrophages changed with most macrophages (>80%) containing less than 10 beads. The dramatic increase in PMN number in the alveolar space after beads and *C.parvum* caused a reduction in the percentage of PMNs with beads from 33% in beads alone group to 4% (Table 12.2). Meanwhile, in the pleural space, PMNs were recruited in relatively high number (29% of the total population) and the total cell number increased nearly three-fold compared to beads alone group (Table 12.1). In spite of such severe pleural inflammation resulting from *C.parvum* in the airspace, negligible numbers of beads could be observed in the pleural leukocytes. It was found that the beads which were seen only very occasionally in cytopins of pleural lavage leukocytes were extracellular, single spheres, being very different to the beads within cells in the alveolar leukocyte cytopins.

The results suggested that there is no direct transfer of compact particles of around 1 μm between the alveolar space and the pleural space, even during acute inflammation. To confirm this hypothesis, we extended our observations to assess numbers of leukocytes containing *C.parvum* organisms in both spaces. Table 12.3 shows that one day after *C.parvum* I/T, numbers of macrophages and PMNs in the alveolar space which were seen to contain organisms were 26% and 34% respectively. By day 5, the percentages of macrophages and PMNs containing organisms dropped to 1 and 0%. Nevertheless, organisms could be found in neither pleural macrophages nor PMNs at any time. These findings indicated that compact particles with diameter of 0.77 μm (the diameter of bead studied) are not likely to be transferred from the alveolar space to the pleural space.

12.3. Secretion of cytokines by pleural leukocytes *in vitro* after I/T *C.parvum*

Normal pleural leukocytes spontaneously produced IL-1 and TNF in culture (Figs 12.3 and 12.4). One day after I/T *C.parvum*, the pleural leukocytes produced less IL-1 and TNF *in vitro* compared to normal leukocytes. Five days after instillation, this reduction was even more marked.

12.4. PAI production by pleural leukocytes *in vitro* after I/T *C.parvum*

Fig 12.5 shows that PAI production by pleural leukocytes one day after I/T *C.parvum* increased significantly compared to control. However, five days after *C.parvum* I/T, PAI activity produced by pleural leukocytes had recovered to normal levels.

12.5. Macrophage and PMN chemotaxis caused by pleural leukocyte supernatant

Supernatants of the pleural leukocytes from rats intratracheally instilled with *C.parvum* had slight chemotactic activity for macrophages compared to

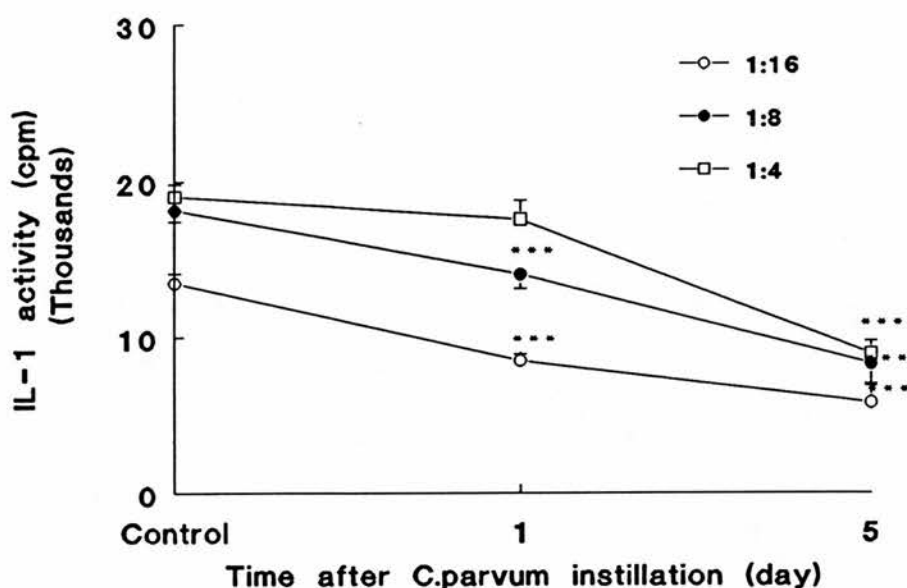


Figure 12.3 IL-1 production by pleural leukocytes at various times after I/T *C. parvum*

Each point represents mean and SEM of 4-9 experiments in triplicate. Asterisk denotes a significant difference from the corresponding dilution of control: ***= $P < 0.001$.

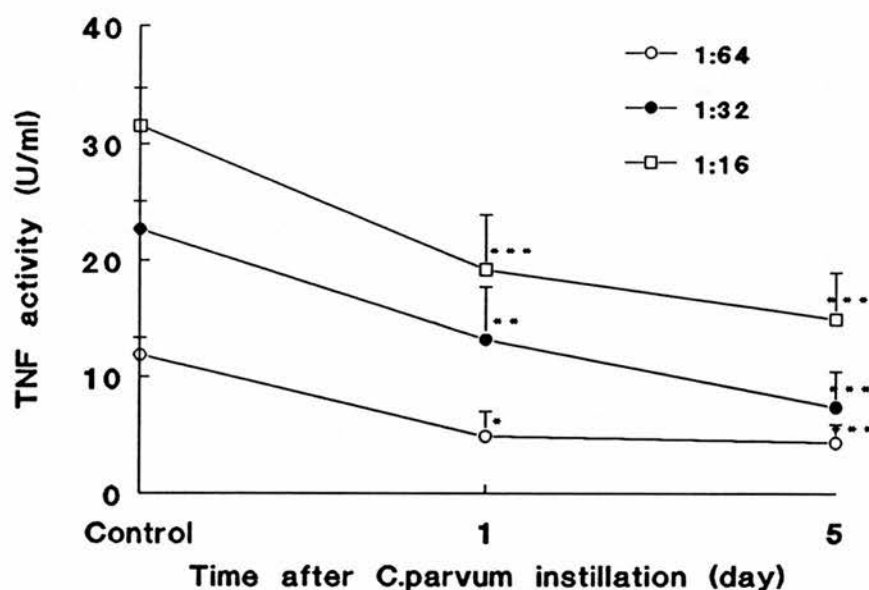


Figure 12.4 TNF production by pleural leukocytes at various times after I/T *C. parvum*

Each point represents the mean and SEM of 4-7 separate experiments performed in triplicate. Asterisk denotes a significant difference from corresponding dilution of control: *= $P < 0.05$, **= $P < 0.01$ and ***= $P < 0.001$.

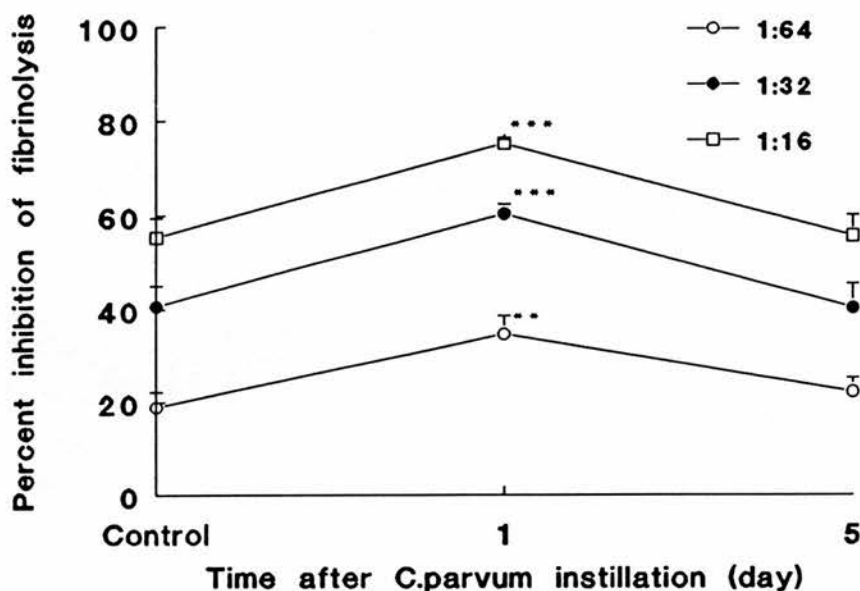


Figure 12.5 Fibrinolysis inhibition caused by pleural leukocyte supernatants at various times after I/T *C. parvum*

Results are presented as mean and SEM of 3–8 separate experiments performed in triplicate. Asterisk denotes a significant difference from corresponding dilution of control: **= $P < 0.01$ and ***= $P < 0.001$.

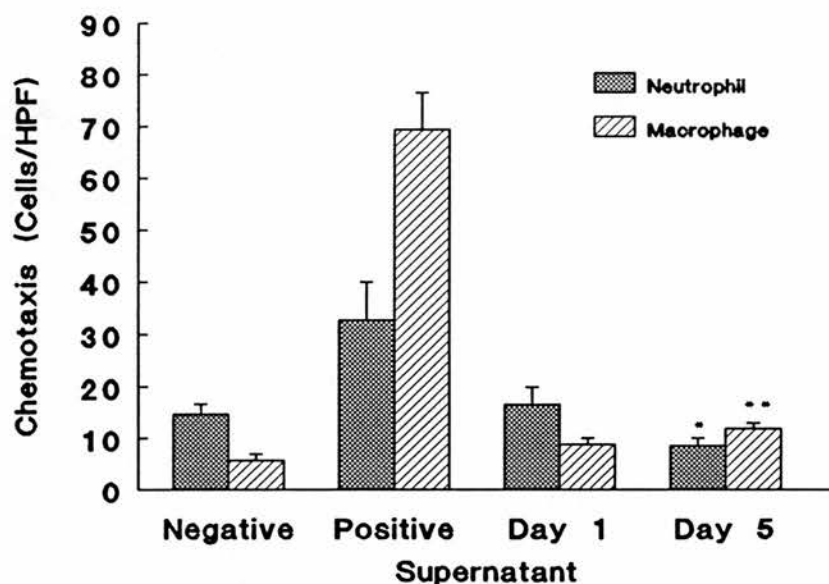


Figure 12.6 Macrophage and neutrophil chemotaxis caused by pleural leukocyte supernatants at various times after I/T *C. parvum*

Each bar shows mean and SEM of 2 to 6 separate experiments. Asterisk denotes a significant difference from negative control: *= $P < 0.05$, **= $P < 0.01$.

negative control (Fig 12.6). This activity increased from day 1 to day 5. There was no PMN chemoattractive activity in pleural leukocyte supernatants, in contrast, supernatants from pleural leukocyte 5 days after I/T *C.parvum* showed reduced ability to attract PMNs compared to negative control.

Table 12.3 ANALYSIS OF C.PARVUM ORGANISMS IN THE ALVEOLAR AND PLEURAL LEUKOCYTES AFTER C.PARVUM I/T

Cells	Percentage of cells with organisms
<u>Alveolar macrophages</u>	
day 1	26 (3.1)
day 5	1 (0.6)
<u>Alveolar PMNs</u>	
day 1	34 (5.6)
day 5	0
<u>Pleural macrophages</u>	
day 1	0
day 5	0
<u>Pleural PMNs</u>	
day 1	0
day 5	0

Results are presented as mean(SD) of 3 to 5 separate experiments.

12.6. Mesothelial cell lysis and detachment caused by alveolar and pleural leukocytes after I/T *C.parvum*

Figure 12.7 shows that one day after *C.parvum* I/T, alveolar leukocytes caused considerable mesothelial cell detachment in an effector:target-related manner; no important mesothelial cell lysis resulted from by the addition of alveolar leukocytes.

Normal pleural leukocytes induced neither mesothelial cell lysis nor detachment (Fig. 7.3). However, one day after *C.parvum* I/T, pleural leukocytes, which contained more than 28% of PMN, dramatically induced detachment of mesothelial cells in culture, but had no influence on mesothelial cell lysis (Fig. 12.8).

Figure 12.9 illustrates that 5 days after *C.parvum* I/T, pleural leukocytes had almost no effect in inducing mesothelial cell detachment, but caused

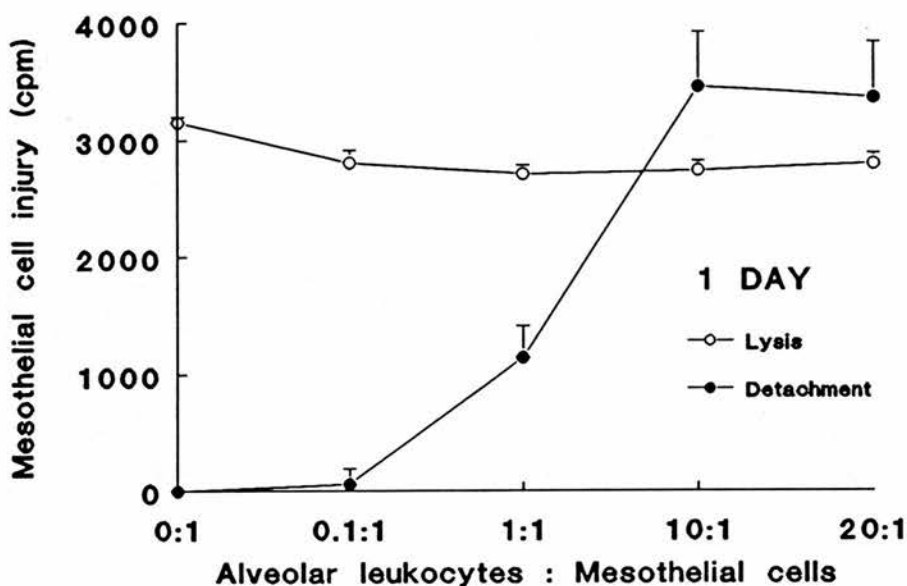


Figure 12.7 Mesothelial cell lysis and detachment injury caused by PVG rat alveolar leukocytes one day after I/T *C.parvum*

Each point represents mean and SEM of 2 to 3 separate experiments performed in triplicate.

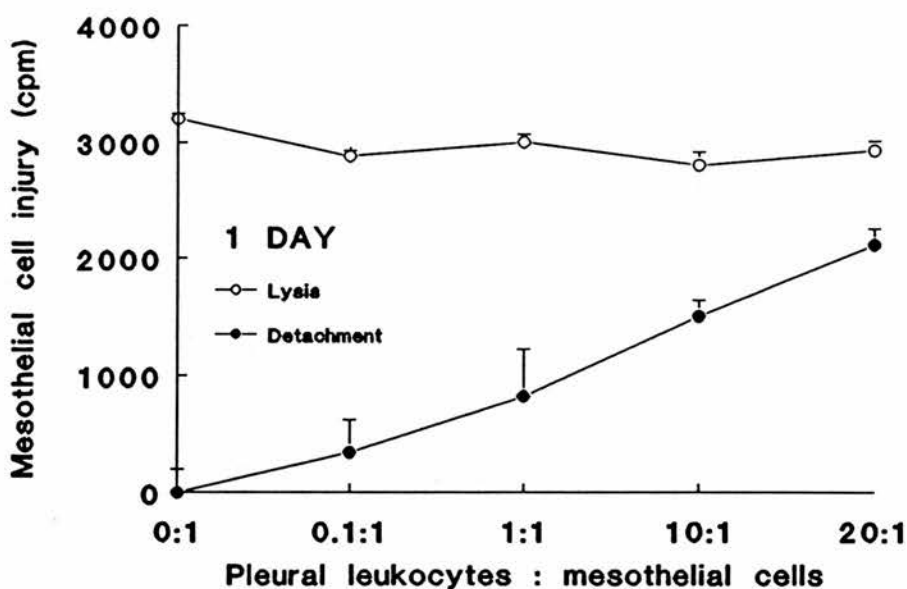


Figure 12.8 Mesothelial cell lysis and detachment injury caused by PVG rat pleural leukocytes one day after I/T *C.parvum*

Each point represents mean and SEM of 2 to 4 separate experiments in triplicate.

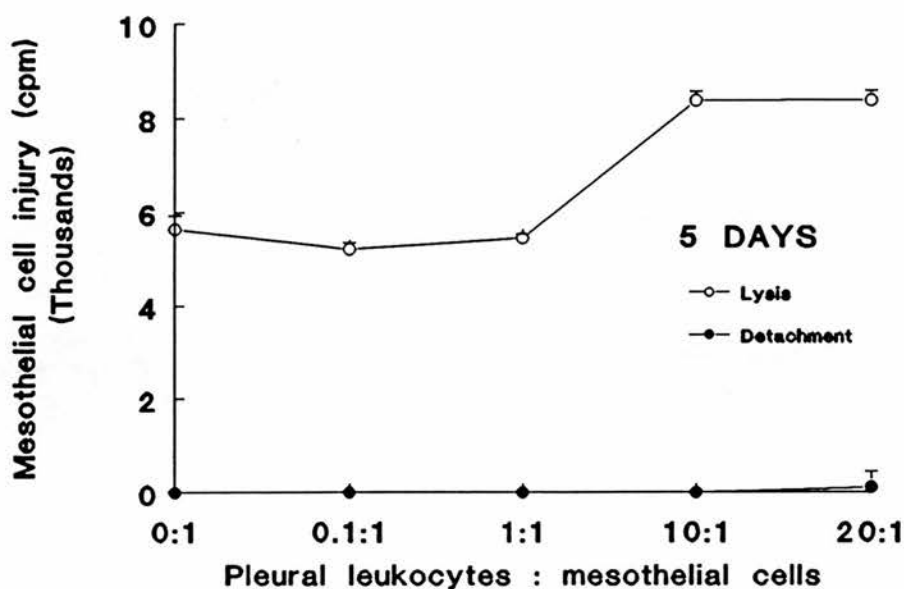


Figure 12.9 Mesothelial cell lysis and detachment injury caused by HAN rat pleural leukocytes 5 days after I/T *C.parvum*

Each point represents mean and SEM of 3 separate experiments in triplicate.

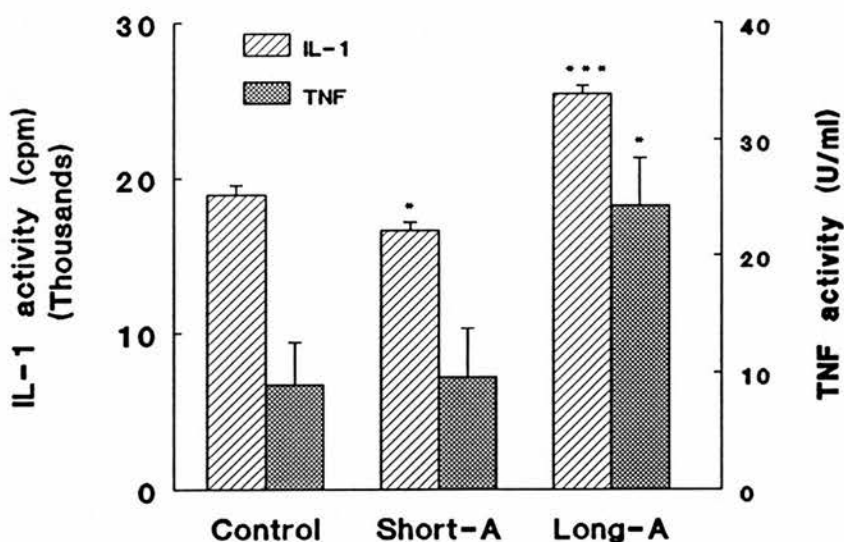


Figure 13.1 TNF and IL-1 production by alveolar macrophages treated with 50 μ g/ml long or short fibre amosite *in vitro*

Each bar stands for the mean and SD of 2-4 separate experiments in triplicate. The dilutions of supernatant for IL-1 was 1:16, and for TNF was 1:256. Abbreviation represent: Contl:- control, Short-A:- short fibre amosite, Long-A:- long fibre amosite. Asterisk denotes a significant difference from control: *=P<0.05, ***=P<0.001.

mesothelial cell lysis at higher rates between the leukocytes and mesothelial cells. Since this experiment was carried out using HAN rats due to shortage of PVG rats, it is difficult to compare this result with the result of Fig. 12.8.

13. Effect of long and short amosite fibre asbestos on cytokine production

The following experiments were carried out using HAN rats.

13.1. TNF and IL-1 production by alveolar macrophages *in vitro* treated with 50 µg/ml short and long fibre amosite asbestos

Normal rat alveolar leukocytes contained more than 95% macrophages (Table 1.1) and are referred to as alveolar macrophages. Table 13.1 shows that normal alveolar macrophages produced TNF and IL-1 in culture and the production was increased significantly when the macrophages were stimulated with 100 ng/ml LPS.

Table 13.1 EFFECT OF LPS STIMULATION ON IL-1 AND TNF PRODUCTION BY ALVEOLAR MACROPHAGES

Cytokine	without LPS	with LPS
IL-1 (cpm)	2782 (108)3	3894 (441)3
TNF (U/ml)	7.40 (3.27)4	11.82 (1.61)4

Results are presented as mean(SD)n of the experiments performed in triplicate. The dilutions of supernatants for IL-1 measurement was 1:16, for TNF measurement was 1:128. There is statistically significant difference between with and without LPS for IL-1 ($P<0.01$) and TNF ($P<0.05$).

Figure 13.1 illustrates evidently that alveolar macrophages stimulated with 50 µg/ml of long amosite produced more TNF in culture than the cells stimulated with short amosite. Short amosite-treated cells produced the same levels of TNF as control macrophages. As demonstrated in Fig. 13.1, long amosite asbestos also stimulated alveolar macrophages to produce more IL-1 activity in culture than short amosite did.

We also utilised two frequently used dust controls, TiO_2 and DQ_{12} , in these experiments. It was found that DQ_{12} stimulated alveolar macrophages *in vitro* to produce increased TNF compared to control (Fig. 13.2). However, TiO_2 had no significant stimulating effect on macrophages.

13.2 Studies on the effect of opsonized dusts on TNF production by alveolar macrophages

In this section, the results were presented as total TNF activity in

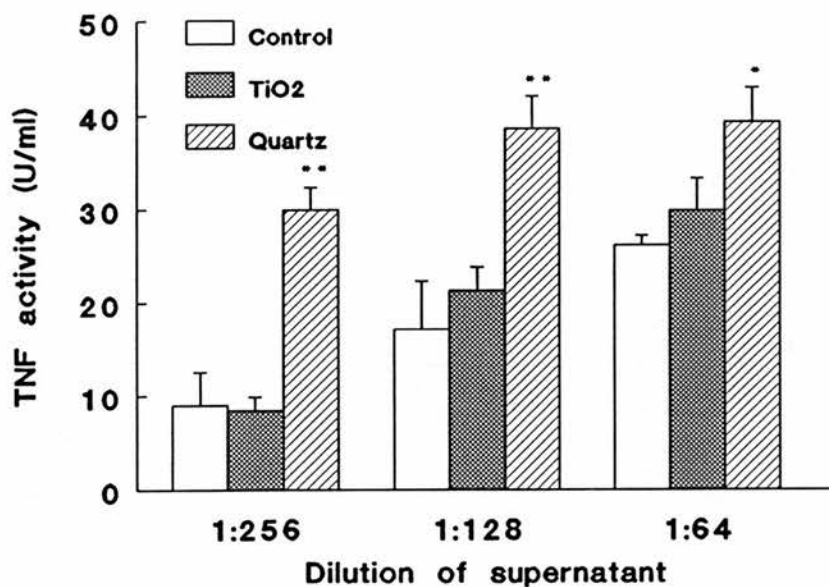


Figure 13.2 TNF production by alveolar macrophages treated with 50 $\mu\text{g/ml}$ of mineral dusts *in vitro*

Each bar stands for the mean and SEM of 2-3 separate experiments performed in triplicate. Asterisk denotes a significant difference from control: *= $P < 0.05$, **= $P < 0.01$.

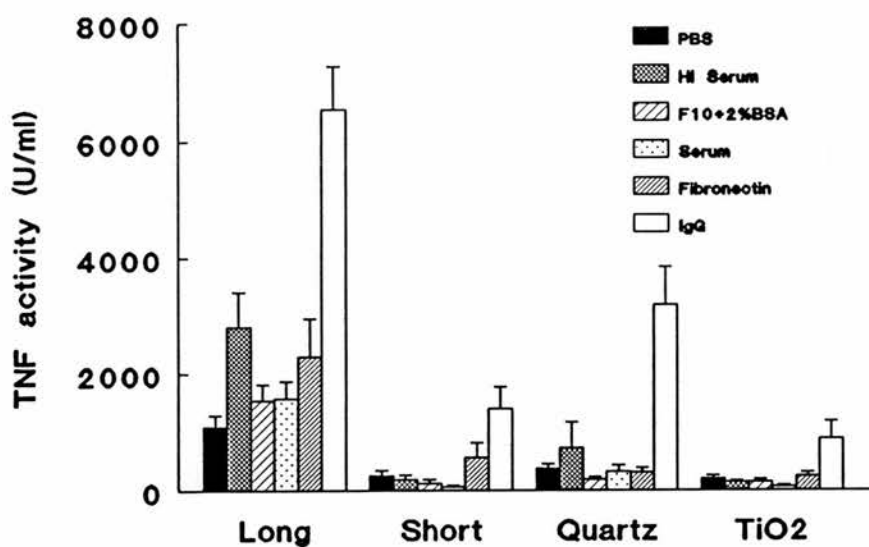


Figure 13.3 Effect of dust opsonization on TNF production by alveolar macrophages *in vitro*

The concentration of rat IgG and fibronectin were 50 $\mu\text{g/ml}$. The dose of dust added to alveolar macrophage cultures was 50 $\mu\text{g/ml}$. Each bar is the mean and SEM of 3 separate experiments performed in triplicate. Abbreviations represent: Long:- long fibre amosite, short:- short fibre amosite, HI serum:- heat-inactivated rat serum.

supernatants rather than TNF activity in diluted supernatants as shown above.

Figure 13.3 shows the effect of the opsonization of dusts on TNF production by alveolar macrophages *in vitro*. Dusts opsonized by PBS (effectively unopsonized) were treated as controls. For long amosite, after opsonization by F10 medium plus 2% BSA and rat serum, TNF-stimulating activity increased slightly. However, when opsonized by heat-inactivated rat serum or fibronectin, the stimulating activity of long amosite was increased moderately. IgG-opsonized long amosite had by far the greatest effect on TNF production by alveolar macrophages compared to other treatments, with the TNF activity increasing up to 6 fold compared to PBS control. Short amosite had a much less stimulatory effect compared to long amosite.

Amongst different opsonins, apparently, IgG treatment caused the biggest enhancement of the capability of short amosite to stimulate macrophages to produce TNF in culture whilst fibronectin treatment was also moderately effective. Comparison between TiO_2 and $\text{DQ}_{1,2}$ indicated that $\text{DQ}_{1,2}$ stimulated more TNF production by macrophages than TiO_2 did and $\text{DQ}_{1,2}$ had much bigger response to the opsonization of IgG than TiO_2 .

Further investigation was carried out to discover the response of different dusts to IgG opsonization. Figure 13.4 shows that long amosite had the most considerable response to IgG opsonization than other dusts and TiO_2 had the least response. With respect to the concentrations of IgG used for opsonization, it was clear that, at lowest dose of opsonizing IgG, i.e., 10 $\mu\text{g}/\text{ml}$, the ability of long amosite in stimulating TNF production by macrophages had been increased to its maximal level. However, other dusts needed to be opsonized by up to 100 $\mu\text{g}/\text{ml}$ of IgG to exert their maximal stimulating effect on TNF production. For TiO_2 an even higher dose of IgG was required.

An effort has been made to identify the possible role of serum in the opsonization of long amosite by IgG. It was found that there was no significant difference between opsonization by IgG plus rat serum and by IgG plus heat-inactivated rat serum, which may exclude the possible interaction between IgG and some heat-labile substances in serum in the opsonization of dusts, such as complements (Fig. 13.5). BSA which was chosen as a negative, parallel control to IgG had no effect in opsonization of the fibre.

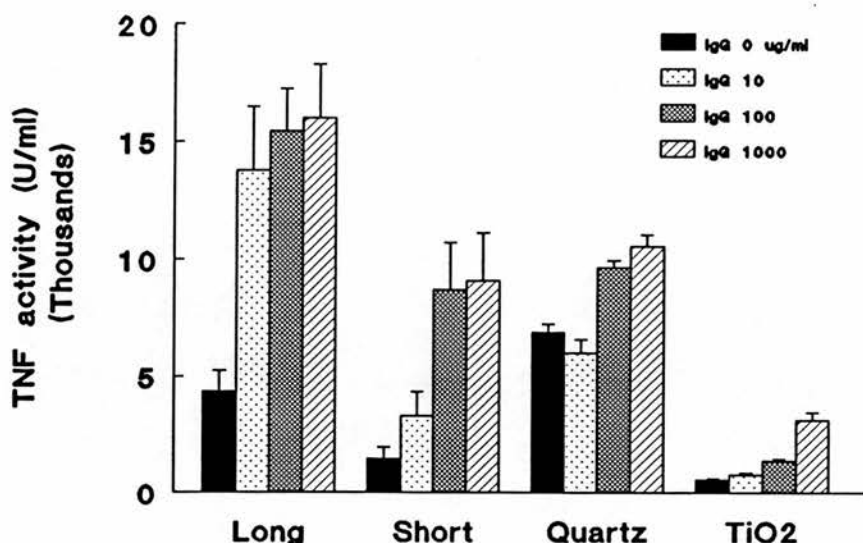


Figure 13.4 Effect of IgG opsonization on the ability of dusts to stimulate TNF production

The concentration of dusts used in study was 50 $\mu\text{g/ml}$. Each bar represents the mean and SEM of 2 separate experiments in triplicate. Abbreviations as for Figure 13.3.

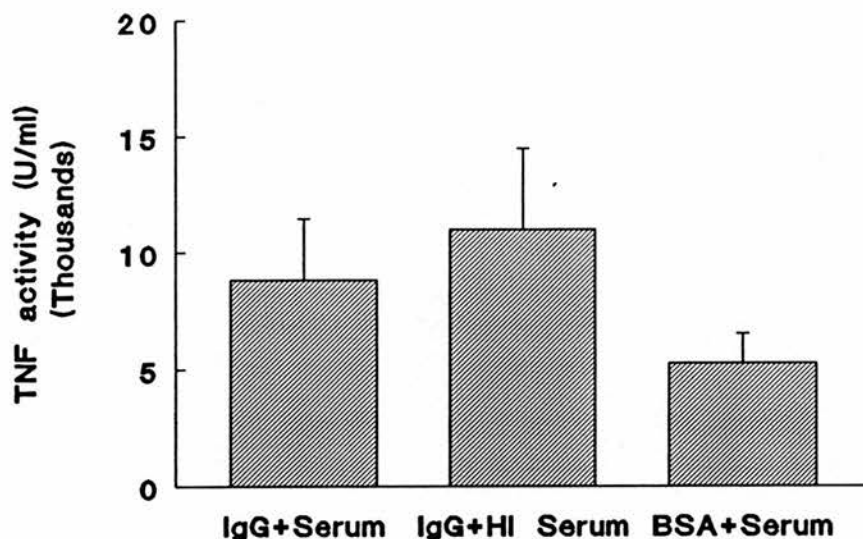


Figure 13.5 TNF production by alveolar macrophages treated with opsonized long fibre amosite

The dust was used in the experiments at a concentration of 50 $\mu\text{g/ml}$. The concentrations of IgG and BSA in the experiments were 50 $\mu\text{g/ml}$. Each bar represents the mean and SEM of 3 separate experiments performed in triplicate.

14. Analysis of beads in bronchoalveolar and pleural leukocytes after rats were intratracheally instilled with the beads and dusts

14.1. Bronchoalveolar and pleural leukocyte components after beads and/or dusts I/T

Table 14.1 shows that total numbers and differential counts of bronchoalveolar leukocytes various times after beads I/T alone or with other dusts. Instillation of bead alone caused moderate acute inflammation in the alveolar space with PMN being 24% of total cells. However, when beads were instilled together with 5 mg of DQ₁₂, a severe inflammation appeared in the alveolar space (more than 74% PMN in the population) with the total cell number increasing 2.5 fold. Five mg of chrysotile together with beads also resulted in severe inflammation (more than 68% PMN), but the total number was low due to probably difficulty in lavaging from asbestos fibre-blocked airways.

Table 14.1 BRONCHOALVEOLAR LEUKOCYTE COMPONENTS AT VARIOUS TIMES AFTER BEADS AND/OR DUSTS I/T

Treatment	Total No. (x 10 ⁶)	Differential percentage		
		Macrophages	PMN	Lymphocytes
1 day after instillation				
Beads alone	7.36(1.26)	75(3.5)	24(2.8)	1(0.7)
Beads+DQ ₁₂	18.58(10.49)	26(12.0)	74(12.0)	0
Beads+Chry	3.41	32	68	0
1 month after instillation				
Beads alone	5.37(1.32)	98(0)	1(1.4)	1(1.4)
Beads+Croc	5.75(0.71)	94(1.4)	3(0)	3(1.4)
Beads+Chry	2.55(0.57)	95(0.7)	4(2.1)	1(1.4)

Results are presented as the mean (SD) of 2 separate experiments except for the group of 1 day beads + chrysotile group which has only 1 experiment due to a mistake in the instillation procedure. Croc: crocidolite; Chry: chrysotile. The dose of dusts were 5 mg per rat. Beads were diluted 1:20 with PBS and 0.2 ml of the solution was injected together with 0.3 ml dust suspension.

At one month post beads and/or dusts I/T, alveolar leukocyte populations had nearly recovered to normal level except for a low level of PMN (Table 14.1).

Table 14.2 illustrates that beads alone or together with dusts I/T did not

substantially affect the components of pleural leukocytes, the only exception was a slight recruitment of PMN one day after beads + DQ₁₂.

Table 14.2 PLEURAL LEUKOCYTE COMPONENTS VARIOUS TIMES AFTER BEADS AND/OR DUSTS I/T

Treatment	Total No	Differential percentage				
	(x 10 ⁶)	Macrophages	PMN	Lymphocytes	Mast-cells	Eosinophils
1 day after instillation						
Beads	4.09(0.70)	76(6.4)	0	1(0)	16(3.5)	7(2.8)
Bead+DQ ₁₂	5.02(1.39)	73(8.5)	4(5.0)	1(1.4)	13(2.1)	9(1.8)
Bead+Chry	4.31	79	0	2	11	8
1 month after instillation						
Beads	3.45(0.64)	82(4.2)	0	1(0.7)	12(5.7)	5(2.1)
Bead+Croc	5.93(1.10)	76(2.8)	0	2(0)	10(1.4)	12(4.2)
Bead+Chry	7.30(0.42)	78(0.7)	0	2(0.7)	8(0.7)	12(0.7)

See footnote to Table 14.1 for detail.

14.2. Counts of beads in alveolar and pleural phagocytes

Table 14.3 shows that there was a high percentage of macrophages with beads present in the alveolar space (72%) and most macrophages had more than 10 beads (71%). However, one day after beads plus DQ₁₂ or chrysotile asbestos I/T, the percentage of macrophages containing beads decreased and more than 60% of macrophages contained less than 5 beads. One month after instillation of beads alone, 53% of macrophages were found to contain beads and the distribution of beads in the cells shifted towards the 1-5 group. The percentage of macrophages with beads decreased in both beads plus crocidolite and beads plus chrysotile groups.

Studies on alveolar PMN showed that 1 day after beads and/or dust I/T, there was a fairly high percentage of PMN-containing beads (20-33%) and most of them had less than 5 beads (90-96%) (Table 14.4). When one month had elapsed after instillation, only a few PMN still existed in the lung and all of them had less than 5 beads per cell.

Table 14.3 COUNTS OF BEADS IN ALVEOLAR MACROPHAGES

Treatment	<u>Cells with beads(%)</u>	<u>Distribution of beads in cells (%)</u>		
		1-5/cell	6-10/cell	>10/cell
1 day after instillation				
Beads	72 (7.1)	12(8.5)	17(4.2)	71(12.7)
Beads+DQ ₁₂	48 (19.8)	64(22.6)	18(5.7)	18(17.0)
Beads+Chry	28	60	20	20
1 month after instillation				
Beads	53 (15.6)	34(2.8)	25(4.2)	41(7.1)
Beads+Croc	39 (7.8)	35(2.8)	24(5.0)	41(2.1)
Beads+Chry	24 (7.1)	51(12.7)	16(0.7)	33(13.4)

See footnote to Table 14.1 for detail.

Table 14.4 COUNTS OF BEADS IN ALVEOLAR PMN

Treatment	Cells with beads(%)	Distribution of beads in cells(%)		
		1-5/cell	6-10/cell	>10/cell
1 day after instillation				
Beads	33 (18.38)	90(2.8)	9(1.4)	1(1.4)
Beads+DQ ₁₂	23 (14.9)	95(7.1)	5(7.1)	0
Beads+Chry	20	96	4	0
1 month after instillation				
Beads	<1	100(0)		
Beads+Croc	8 (3.5)	100(0)		
Beads+Chry	6 (0)	100(0)		

See footnote to Table 14.1 for detail.

Tables 14.5 and 14.6 indicated that there were almost no macrophages or PMN containing beads in the pleural space. Beads were found only very occasionally and there were free.

Table 14.5 COUNTS OF BEADS IN PLEURAL MACROPHAGES

Treatment	Cells with beads(%)	Distribution of beads in cells(%)		
		1-5/cell	6-10/cell	>10/cell
1 day after instillation				
Beads	<1	100	0	0
Beads+DQ ₁₂	<1	100	0	0
Beads+Chry	0	0	0	0
1 month after instillation				
Beads	0	0	0	0
Beads+Croc	0	0	0	0
Beads+Chry	0	0	0	0

See footnote to Table 14.1 for detail.

Table 14.6 COUNTS OF BEADS IN PLEURAL PMN

Treatment	Cells with beads(%)	Distribution of beads in cells(%)		
		1-5/cell	6-10/cell	>10/cell
1 day after instillation				
Beads	0	0	0	0
Beads+DQ ₁₂	<1	100	0	0
Beads+Chry	0	0	0	0
1 month after instillation				
Beads	0	0	0	0
Beads+Croc	0	0	0	0
Beads+Chry	0	0	0	0

See footnote to Table 14.1 for detail.

Tables 14.7 and 14.8 shows the results of an experiment done incidentally. By mistake during the procedure of intratracheal instillation, part of the suspension of beads and chrysotile asbestos was injected into pleural space. Table 14.7 shows extensive recruitment of pleural leukocytes (32.55×10^6 , six times more than other treatments) with nearly 70% of cells being PMN. Bead counting in the pleural leukocytes confirmed mis- introduction of beads into pleural space,

which gave a totally different picture from the normal intratracheal instillation studies.

Table 14.7 ALVEOLAR AND PLEURAL LEUKOCYTE COMPONENTS 1 DAY AFTER INTRATRACHEAL AND INTRAPLEURAL INSTILLATION OF BEADS AND CHRYSOTILE ASBESTOS

Total No (x 10 ⁶)	Differential percentage				
	Macrophages	PMN	Lymphocytes	Mast-cells	Eosinophils
Alveolar					
0.9	89	9	2		
Pleural					
32.55	30	69	0	1	0

Results of an experiment with mixed intrapleural and intratracheal injection of 5 mg chrysotile and beads.

Table 14.8 COUNTS OF BEADS IN ALVEOLAR AND PLEURAL MACROPHAGES AND PMN

Cell with beads (%)	<u>Distribution of beads in cells (%)</u>		
	1-5/cell	6-10/cell	>10/cell
Alveolar macrophages			
2	60	22	18
Alveolar PMN			
7	84	8	8
Pleural macrophages			
8	88	10	2
Pleural PMN			
<1	92	4	4

Result of an experiment with mixed intrapleural and intratracheal instillation of beads and 5 mg of chrysotile.

15. Transmission electron microscopy study on pleural leukocytes and pleural structure

15.1. Transmission electron microscopy study on the possibility of transfer of asbestos fibre from airspace to pleural space

Transmission electron microscope studies had been carried out to determine whether asbestos fibre in the airspace could reach the pleural space. Pleural leukocytes were examined carefully for the presence of fibres. Both long or short term asbestos inhalation and intratracheal instillation studies were carried out. The inhalation studies included HAN rats exposed to 10 mg/m^3 chrysotile asbestos from 1, 3, 4, 6 months till 1 year, exposed to 10 mg/m^3 chrysotile and 2 mg/m^3 quartz together, 10 mg/m^3 amosite and 2 mg/m^3 quartz together or 10 mg/m^3 long fibre amosite and 2 mg/m^3 quartz for one year.

The results of light microscope studies on pleural leukocyte components after rats were inhaled different asbestos or with other dusts were shown in Table 15.1. It was clear that different time after 10 mg/m^3 chrysotile asbestos inhalation, no PMN could be found in pleural leukocyte populations. There was no considerable difference among various time groups. The inhalation of chrysotile or amosite together with quartz caused slight recruitment of PMN in the pleural leukocyte populations, especially, in 10 mg/m^3 long fibre amosite together with 2 mg/m^3 quartz group in which PMN accounted for 6% of total cell number. In addition, the combination of chrysotile and quartz, amosite and quartz or long fibre amosite and quartz in inhalation study resulted in the increase of pleural leukocyte numbers. However, when chrysotile alone groups were compared with other treatment groups, it was noted that the percentage of eosinophils increased remarkably apart from 1 year term inhalation study.

Table 15.1 PLEURAL LEUKOCYTE COMPONENTS AFTER RATS INHALED ASBESTOS ALONE OR TOGETHER WITH QUARTZ

Total No. ($\times 10^6$)	Differential percentage				
	Macrophages	PMN	Lymphocytes	Mast-cells	Eosinophils
Control					
9.31(4.91)7	73(5.5)7	0	3(3.1)7	17(3.9)7	7(2.6)7
Chrysotile alone					
1 month					
7.78(1.46)2	69(4.2)2	0	2(1.4)2	14(3.5)2	15(2.1)2
3 months					
10.63(0.18)2	71(9.9)2	0	1(0.7)2	15(7.1)2	13(3.5)2
4 months					
11.16(4.61)4	71(4.9)4	0	1(0.5)4	15(3.4)4	13(3.9)4
6 months					
9.38(2.34)2	67(4.2)	0	2(0.7)2	19(2.8)2	12(0.7)2
1 year					
5.61(1.26)2	72(0)2	0	2(0)2	18(5.7)2	8(5.7)2
Chrysotile or amosite + quartz					
Chrysotile + quartz, 1 year					
14.69(5.88)6	74(10.1)6	1(2.0)6	8(8.6)6	8(6.1)6	9(7.4)6
Amosite + quartz, 1 year					
16.31(16.62)3	83(11.6)3	1(0.6)3	1(1.0)3	8(7.4)3	7(3.5)3
Long fibre amosite + quartz, 1 year					
17.55(9.05)5	81(9.7)5	6(11.2)5	5(8.1)5	4(4.4)5	4(2.6)5

Results are presented as mean (SD) n. There is statistically a significant difference in total cell numbers between control v $10\text{mg}/\text{m}^3$ long fibre amosite and in differential percentage of eosinophils between control v all of chrysotile alone groups ($P<0.05$ – $P<0.01$) except for the 1 year group.

Table 15.2 indicates that compared to the pleural leukocytes, bronchoalveolar leukocyte components showed a dramatic change in response to asbestos inhalation, characterised by substantial recruitment of PMN.

Pleural leukocytes were studied by transmission electron microscopy in an attempt to find asbestos fibres in the leukocytes, especially, macrophages. From each treatment up to five blocks were prepared and, in each block more than

100 cells were observed. After rat inhalation exposure to asbestos for various time, pleural macrophages appeared very active (Figs. 15.1 - 15.3) compared to normal pleural macrophages (Fig. 1.2), but no asbestos fibres could be observed in pleural leukocytes. In the same animals, asbestos fibres were easily identified in alveolar macrophages (Figs. 15.4 - 15.7).

Table 15.2 BRONCHOALVEOLAR LEUKOCYTE COMPONENTS VARIOUS TIME AFTER INHALATION OF CHRYSOTILE ASBESTOS

Total No. ($\times 10^6$)	Differential percentage				
	Macrophages	PMN	Lymphocytes	Mast-cells	Eosinophils
Chrysotile 1 month					
7.13(3.05)2	86(6.4)2	13(6.4)2	1(0)2	0	0
Chrysotile 3 months					
6.71(3.13)2	56(15.6)2	40(11.3)2	4(4.2)2	0	0
Chrysotile 4 months					
7.40(1.72)4	66(9.0)4	33(9.0)4	1(0)4	0	0

Results are presented as mean(SD)n.

15.2. Electron microscope study of rat pleura

Figure 15.8 shows an area of control rat visceral pleura. The visceral pleural was found to be composed of a mesothelium separated by a continuous, elastic lamina from the juxta-pleural alveoli or terminal bronchiole (Figs. 15.8, 15.9). There were tight junctions in the mesothelium (Fig. 15.10). Bundles of collagen fibres were observed in submesothelial tissue. Mast cells were found frequently in these areas (Fig. 15.11).

The parietal pleura was composed of a monolayer of mesothelial cells separated from the parietal muscular layer by a thick connective tissue layer. The mesothelial cells were flatter than those of the visceral pleura (Fig. 15.12). Collagen bundles were seen frequently.

15.3. Observation on the pleural structure after intratracheal instillation of *C.parvum* or chrysotile asbestos

Figure 15.13 shows rat visceral pleura one day after intratracheal instillation of 5 mg chrysotile asbestos. There is a accumulation of PMN in alveoli, and meanwhile, mesothelial cells became very active with much more

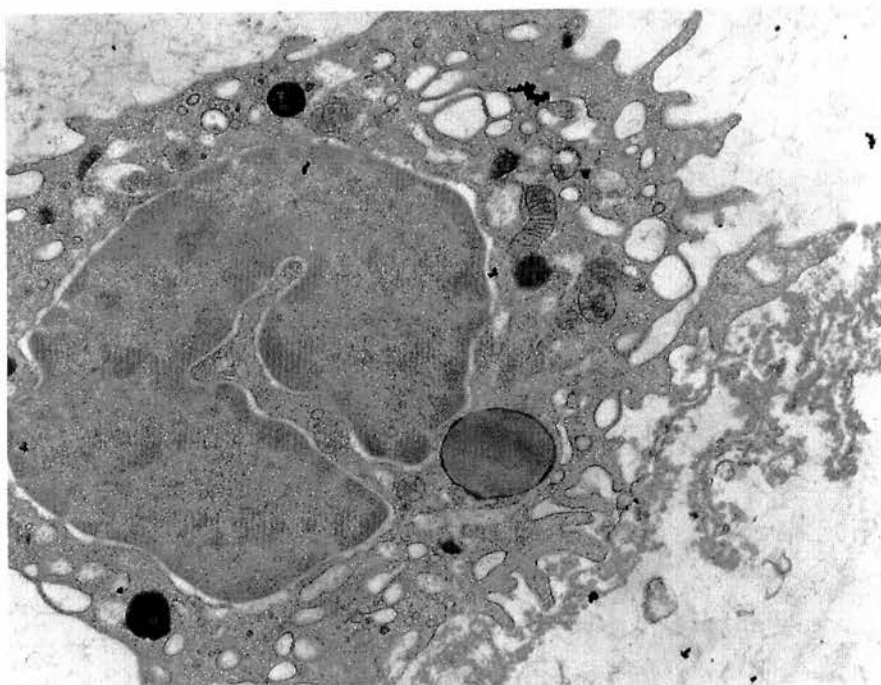


Figure 15.1 Transmission electron micrograph of a pleural macrophage from a HAN rat exposed, by inhalation, to chrysotile asbestos and quartz for one year (x9,000)

Pleural leukocytes were lavaged from a rat one year after cessation of inhalation exposure to 10 mg/m³ chrysotile asbestos plus 2 mg/m³ quartz for one year.

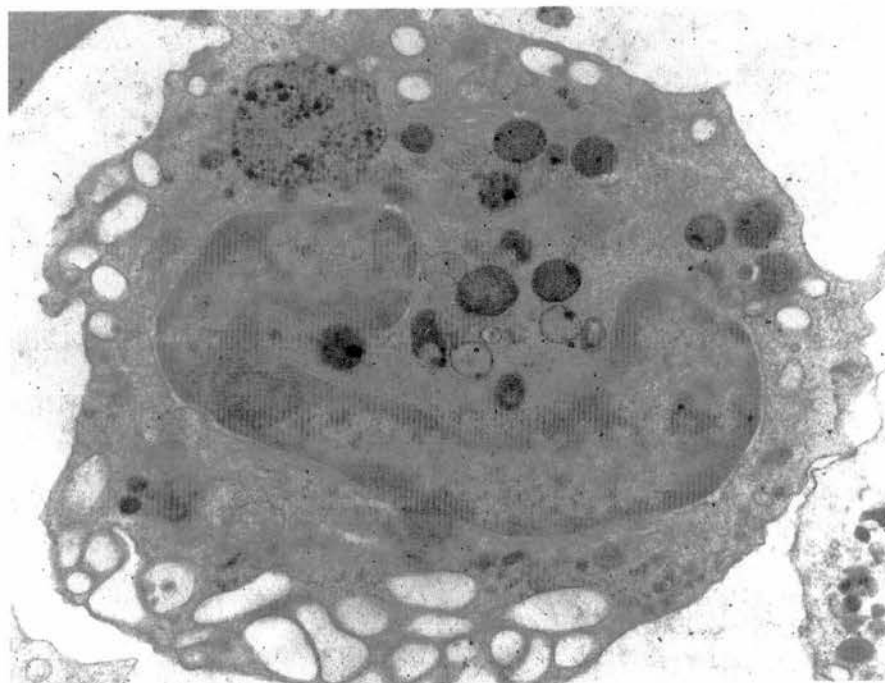


Figure 15.2 Transmission electron micrograph of a pleural macrophage from a HAN rat inhaling chrysotile asbestos and quartz for one year (x8,000)

Pleural leukocytes were lavaged from a rat one year after cessation of inhalation exposure to 10 mg/m³ chrysotile asbestos plus 2 mg/m³ quartz for one year.

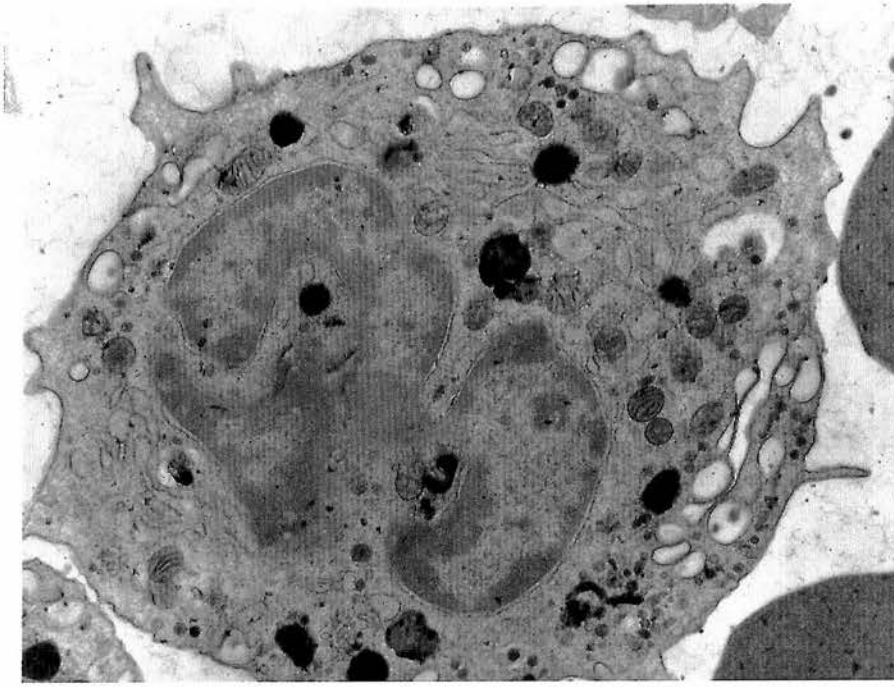


Figure 15.3 Transmission electron micrograph of a pleural macrophage from a HAN rat which had been inhaling chrysotile asbestos and quartz for one year (x8,000)

Pleural leukocytes were lavaged from a rat one year after cessation of inhalation exposure to 10 mg/m³ chrysotile asbestos plus 2 mg/m³ quartz for one year.

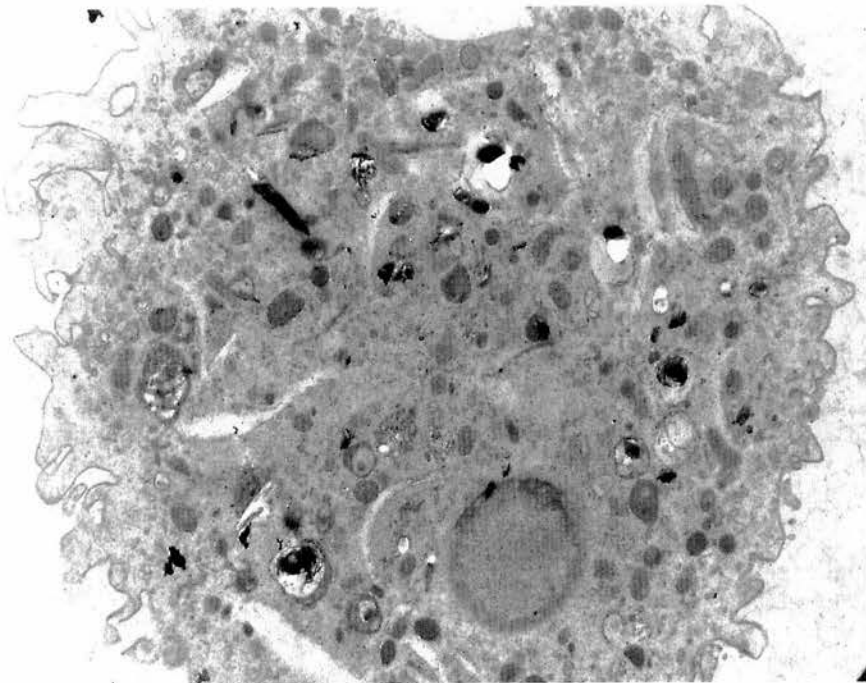


Figure 15.4 Transmission electron micrograph of an alveolar macrophage from a HAN rat inhaling chrysotile asbestos for one month (x5,000)

Alveolar leukocytes were lavaged from a rat inhaling 10 mg/m³ of chrysotile for month. Chrysotile fibrils can be identified within the macrophage.

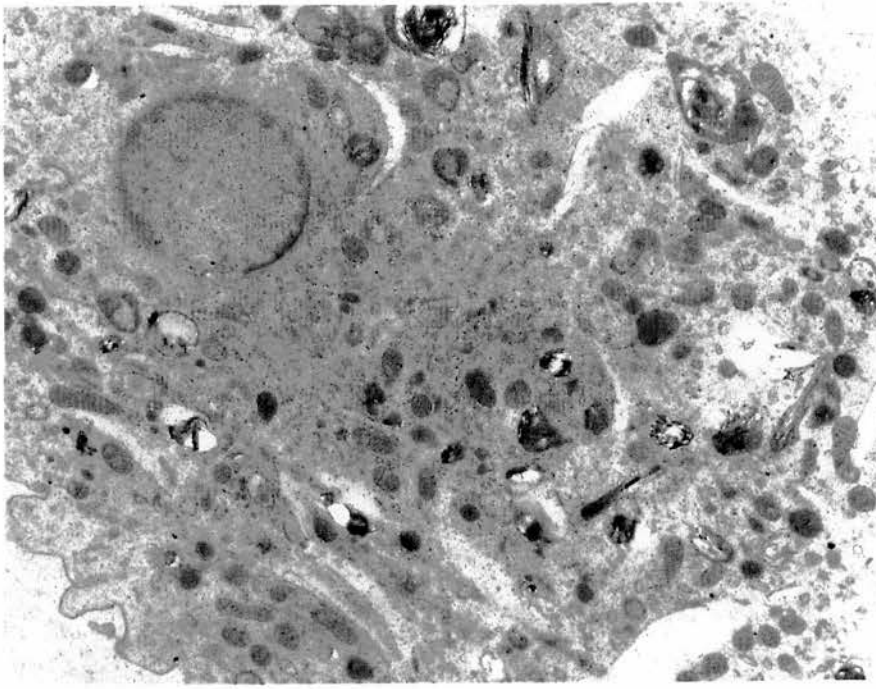


Figure 15.5 Transmission electron micrograph of an alveolar macrophage from a HAN rat inhaling chrysotile asbestos for one month (x6,000)

Alveolar leukocytes were lavaged from a rat inhaling 10 mg/m³ of chrysotile for month. The picture shows chrysotile fibrils in the cytoplasm and phagosomes of the macrophage.

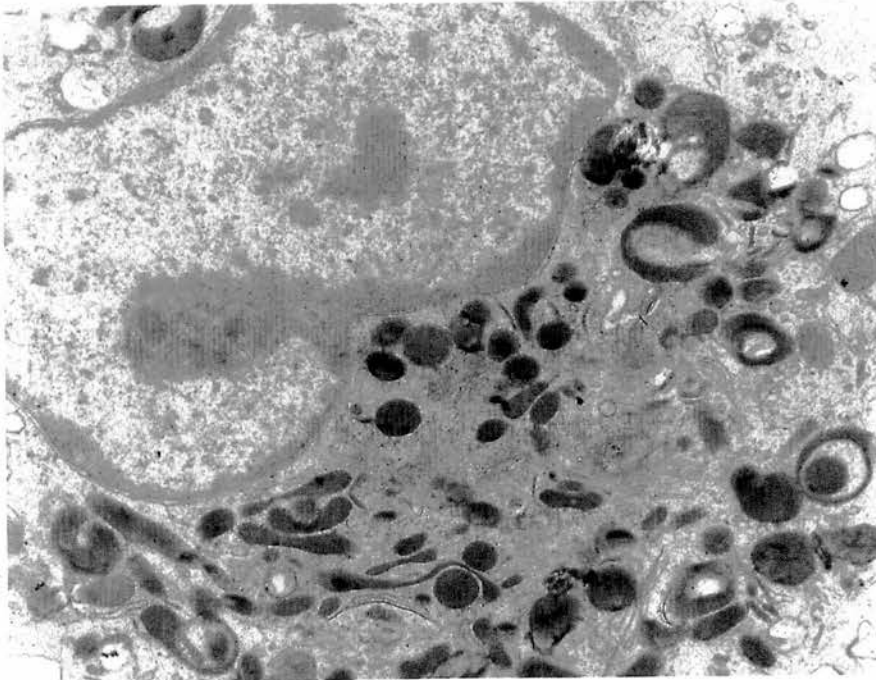


Figure 15.6 Transmission electron micrograph of an alveolar macrophage from a HAN rat inhaling chrysotile asbestos for three months (x9,000)

Alveolar leukocytes were lavaged from a rat inhaling 10 mg/m³ chrysotile for three months. Chrysotile fibrils are visible in phagosomes and cytoplasm of the macrophage.

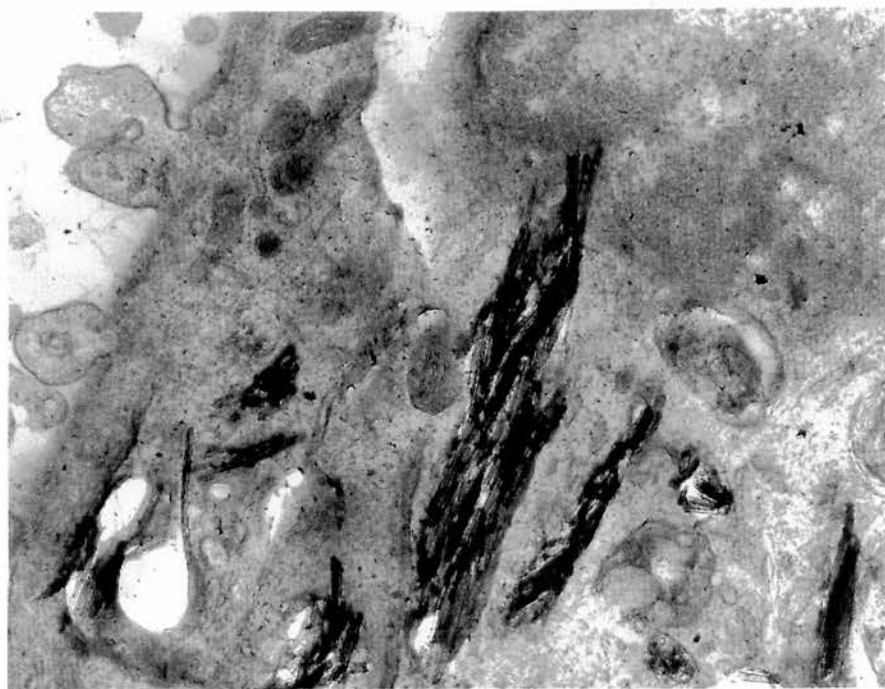


Figure 15.7 High magnification transmission electron micrograph of chrysotile fibrils within an alveolar macrophages (x17,000)

The picture shows part of an alveolar macrophage from a rat which had been inhaling 10 mg/m^3 of chrysotile for one month. Chrysotile fibrils are present in the cytoplasm of the macrophage.

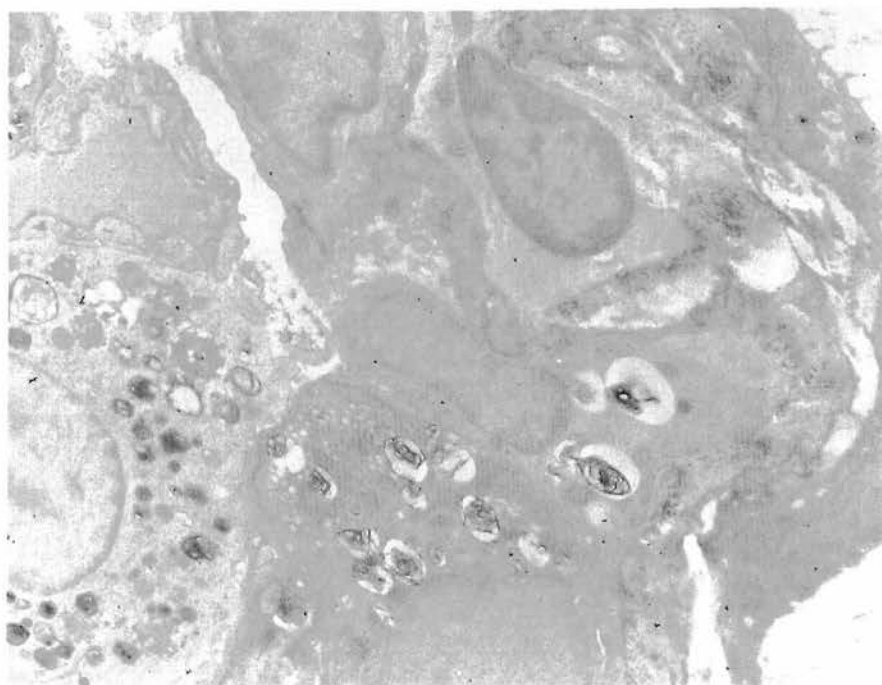


Figure 15.8 Electron micrograph of rat visceral pleura and its subpleural structures (x4,000)

Mesothelial cells with a few microvilli and alveoli can be observed.

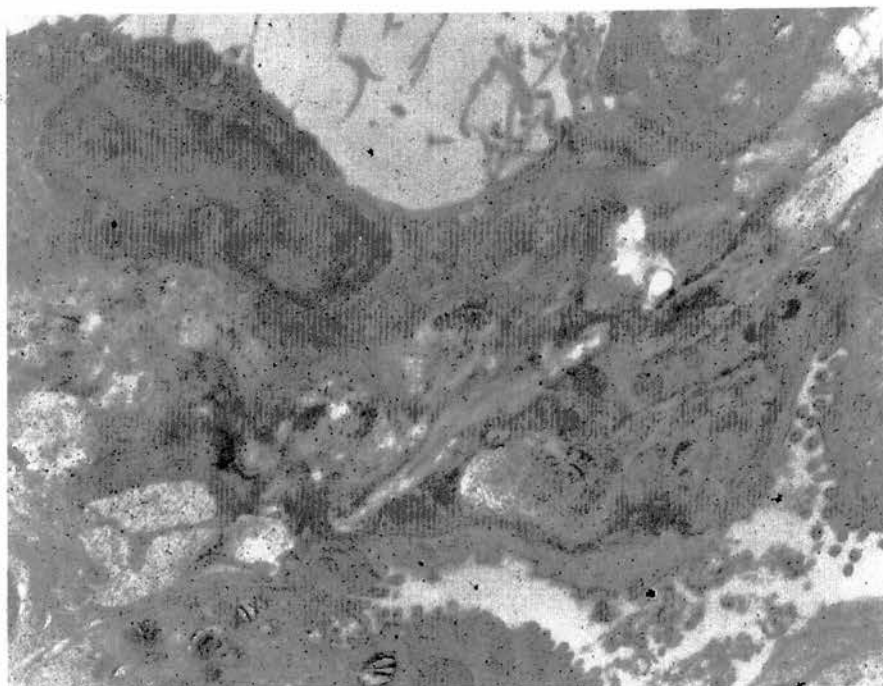


Figure 15.9 Electron micrograph of rat visceral pleura and subpleural structure (x6,000)

A relatively thick mesothelium plus a terminal bronchiole can be observed.

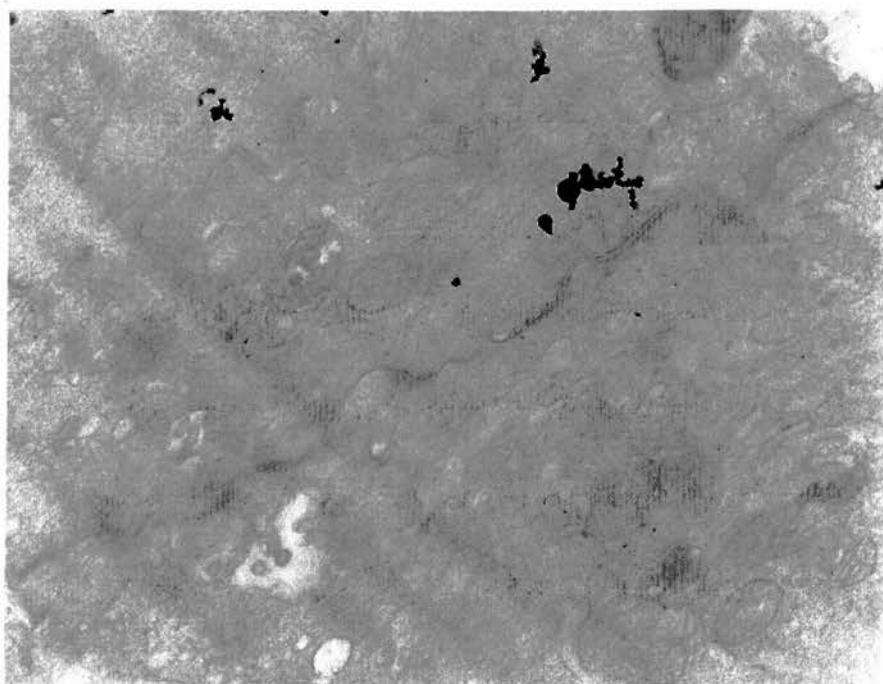


Figure 15.10 Junction between two mesothelial cells of the visceral pleura (x15,000)

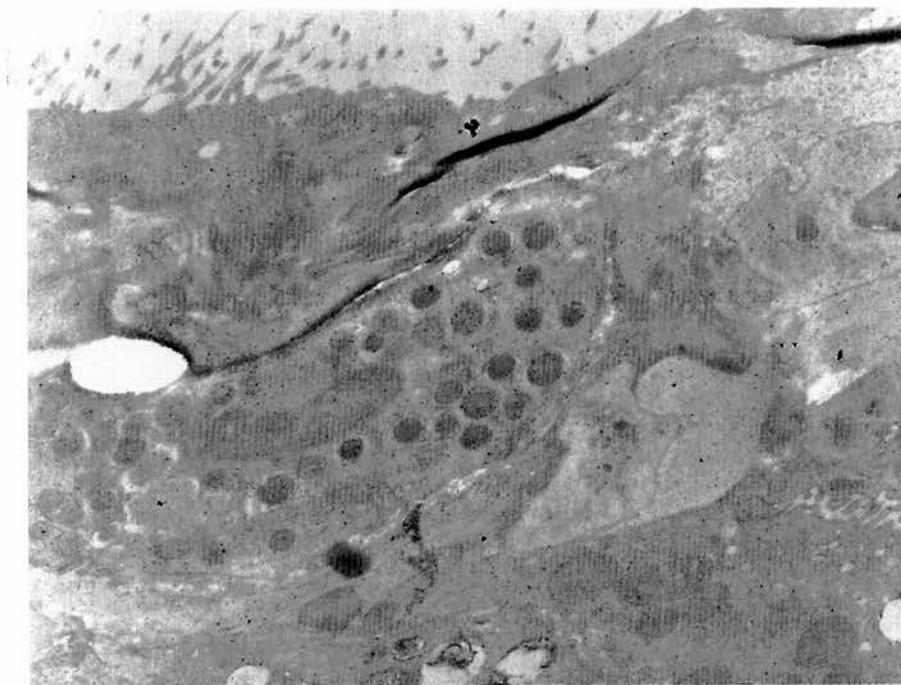


Figure 15.11 Transmission electron micrograph of rat visceral pleura and the subpleural structures (x6,000)

A mast cell is present in the subpleural connective tissue.



Figure 15.12 Transmission electron micrograph of rat parietal pleura (x12,000)

The mesothelium is flatter than visceral mesothelium and is separated, by a connective tissue layer, from the parietal muscular layer (bottom left).

mitochondria in the cytoplasm. It is common that mesothelial cells are capable of proliferating after being injured. Figure 15.14 shows mesothelial debris which is presumed to be exfoliated cell from the pleura after the mesothelium was damaged by the presence of asbestos in alveoli.

Figure 15.15 displays, apparently, the migration of PMN from lung interstitium towards pleural space one day after intratracheal instillation of 1.4 mg *C.parvum*. This may explain the source of PMN found in pleural space during *C.parvum*-induced acute inflammation.

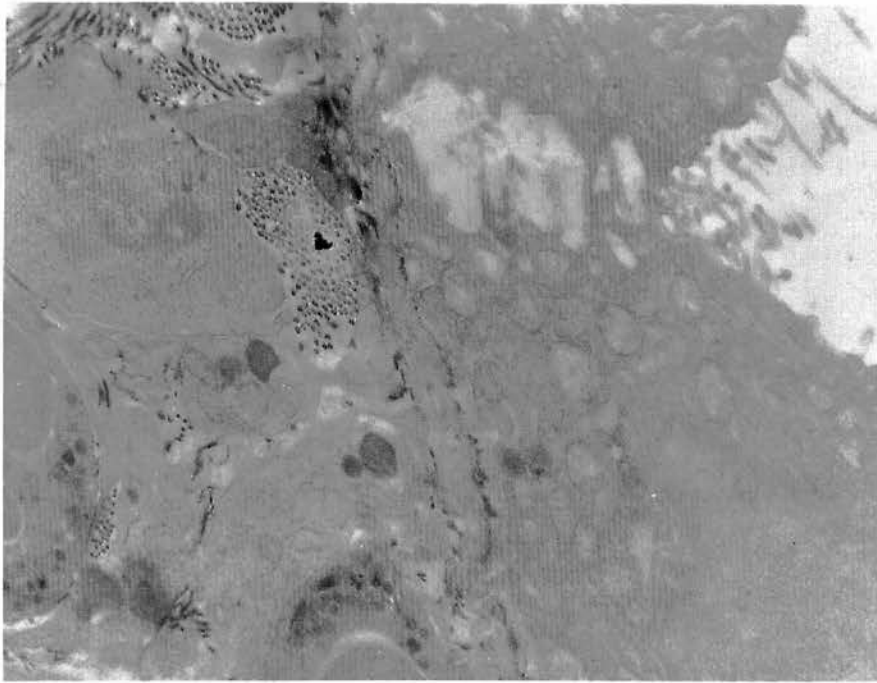


Figure 15.13 Transmission electron micrograph of rat visceral pleura one day after I/T 5 mg chrysotile asbestos (x8,000)

A neutrophil is present in the subpleural connective tissue. Mesothelial cells are rich in mitochondria.

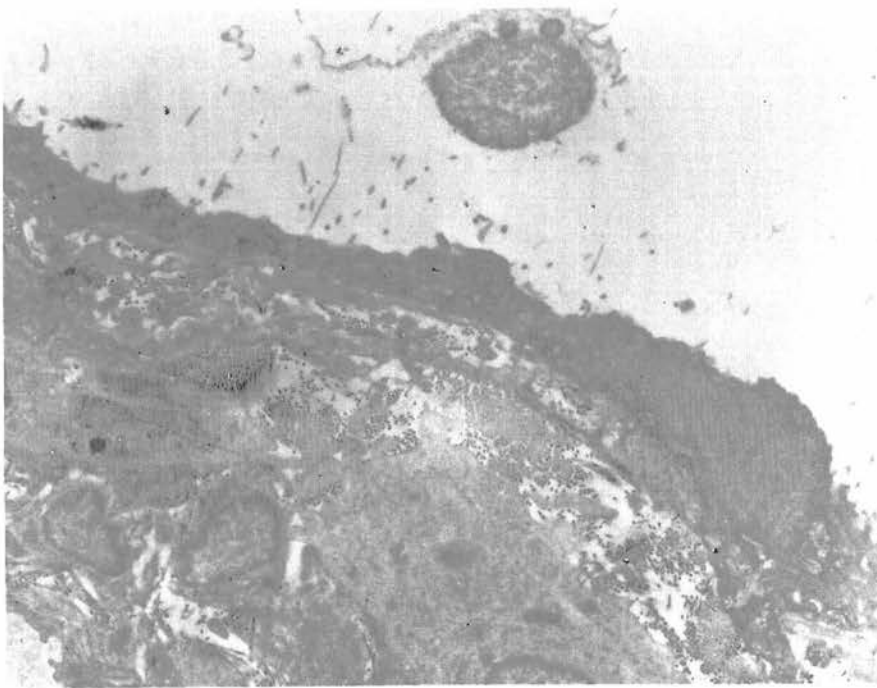


Figure 15.14 Transmission electron micrograph of rat visceral pleura one day after T/T 5 mg chrysotile asbestos (x6,000)

An exfoliated mesothelial cell is present in the pleural space.

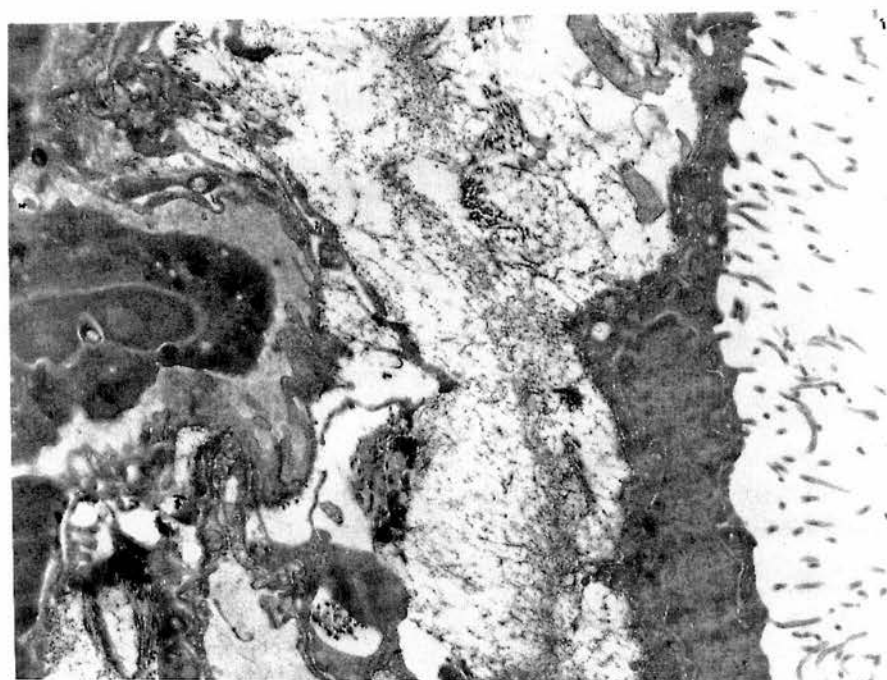


Figure 15.15 Transmission electron micrograph of rat visceral pleura one day after I/T *C.parvum* (x6,000)

A neutrophil from the lung interstitium is apparently migrating towards the pleural space.

DISCUSSION

This thesis has attempted, using a rat model, to elucidate the mechanisms underlying the pathological effects arising in the pleura, from the deposition of asbestos in airspaces. In particular, the effects caused by asbestos deposited in the airspaces on the activities of pleural leukocytes has been studied. The approaches which were taken included: 1). the determination of inflammatory mediator production by pleural leukocytes after intratracheal instillation of asbestos; 2). the possibility of transfer of asbestos fibres from the airspace to the pleura; 3). interactions between pleural leukocytes from asbestos-instilled rats and pleural mesothelial cells *in vitro*; 4). comparison of bronchoalveolar leukocytes and pleural leukocytes after intratracheal asbestos; 5). limited studies in pleural leukocytes from rats which had been inhaling asbestos. The results from this model suggest that deposition of asbestos in the airspaces has important influences on the pleural leukocyte population without substantial transfer of fibre to the pleural space.

1. Fibrinolysis in the pleural space and the effect of intratracheally instilled asbestos

1.1. Normal pleural leukocytes and fibrinolysis

1.1.1. Normal pleural leukocyte population

The present work shows that the numbers of pleural leukocyte are 4.5×10^6 and 8.7×10^6 in normal rats of the PVG and HAN strains respectively. These figures are similar to the observation of Oberdoerster *et al* (1983) that total cell counts in pleural lavages of rats were from 7 to 13×10^6 . In these populations, macrophages account for the majority, which are more than 80% in PVG rats and 70% in HAN rats. The normal pleural leukocytes also contained substantial proportions of mast cells and eosinophils. High numbers of eosinophils and mast cells were also found by Lehnert *et al* (1985), Oberdoerster *et al*, (1983) and Miller *et al* (1989) in the pleural lavage of rats.

Polymorphonuclear leukocytes (PMN) were not normal residents of the pleural space in the present study. In contrast, Oberdoerster *et al* (1983) found that PMN comprised 26–45% in control pleural lavages. Miller *et al* (1989) described some not easily distinguishable cells, with doughnut shaped nuclei, as juvenile PMN or immature monocytes (45–60%) in air-exposed rats. No difficulty was experienced in identifying PMN from other cells in pleural lavages. PMN are characterized by their multi-lobed nucleus confirmed by light microscopy as well as electron microscopy observation. We believe that, normally, PMN appear only during an inflammatory response where they exert important defensive functions. They would not be anticipated to be present as normal residents of the pleural space. Zlotnic *et al* (1982) and Pitt's (1985) work showing none, or less than 1%,

of PMN in normal mice pleural lavages supports this contention.

Mesothelial cells were virtually absent from our normal rat pleural leukocyte preparations, confirming Spriggs and Boddington's (1968) observation that mesothelial cells are not evident in washings from normal serous surfaces.

1.1.2. Normal pleural leukocytes produce plasminogen activator inhibitor (PAI) in culture

Plasminogen activators can convert plasminogen into its active form, plasmin which is involved in the breakdown of fibrin and directly or indirectly promotes degradation of all components of the extracellular matrix (Blasi *et al*, 1987). The activity of plasminogen activators (PA) are regulated by specific inhibitors so that the regulation of the production of plasminogen activator inhibitors (PAI) will be important in matrix metabolism and especially in fibrinolysis. The serous surfaces, such as the pleura, because of their important function in allowing tissues to move smoothly over one another, must maintain efficient fibrinolysis.

It has been shown that bronchoalveolar leukocytes and mesothelial cells activate plasminogen via synthesis of PAs (Donaldson *et al*, 1988_c; Harold *et al*, 1988), and this was also confirmed in the present study. However, pleural leukocytes, cultured *in vitro*, do not activate plasminogen. In contrast, when either alveolar leukocytes or mesothelial cells were cultured together with pleural leukocytes or leukocyte supernatants, their fibrinolytic activity was inhibited. We hypothesized that the inhibitory activity was caused by a substance (inhibitor) secreted by pleural leukocytes.

In an attempt to find out the point of action of the inhibitor, which could operate at the level of the plasminogen activator or plasmin, different model fibrinolysis systems were used to identify the inhibitory substance secreted by pleural leukocytes. Although the pleural leukocyte-derived inhibitor inhibited fibrinolysis by plasmin to a certain degree, this was much less than the inhibitory activity shown against fibrinolysis mediated by urokinase and plasminogen, which could be up to 100% of the total. From the data it is concluded that pleural leukocytes produce a plasminogen activator inhibitor that operates mainly against urokinase-type PAs but also has some activity against plasmin. The finding that the inhibitor substantially blocked the proteolytic activity of urokinase was consistent with other workers' findings: the PAI secreted by human macrophages (Saksela *et al*, 1985) and PAI in ascitic fluid in an experimental tumour (Dong *et al*, 1988) inhibited urokinase but had no activity on tissue-type PA.

PAI from different cell lines have different heat stabilities. In the case of

human macrophage-derived PAI, heat-treatment at 56°C for 30 min abolished its functional activity against urokinase (Chapman and Stone, 1985_b). The rat pleural leukocyte PAI demonstrated in the present studies was heat stable at 56°C for 1 hr but was completely inactivated after incubation at 70°C for 1 hr and so was similar to that described by Saksela *et al* (1985). The inhibitor is greater than 10,000 D which is consistent with general finding that PAI have a molecular mass around 40–50 kD (Sprenger and Kluft, 1987) rising to greater than 100 kD if complexes with proteases are formed (Hart and Rehemtulla, 1988).

Kopitar (1981) has shown evidence that a urokinase inhibitor isolated from pig leukocytes is stable in the pH region from 3.0 to 8.0. In another study, they isolated a human leukocyte urokinase inhibitor (Kopitar *et al*, 1985) and found that the inhibitor was stable from pH 7 to 9, but was inactivated by acid pH and above pH 9. Its activity was progressively lost with decreasing pH. The present study on acid treatment of the inhibitor indicated that the rat PAI is acid unstable and loses its activity at pH 4.0.

In keeping with a large body of evidence showing that the production of PAIs is predominantly by cells of the mononuclear phagocyte lineage (Vassalli and Dayer, 1984; Chapman and Stone, 1985_a; Saksela *et al*, 1985), especially for PAI-2, the present study confirmed that the cellular source of the PAI was the pleural macrophages. By separating the pleural leukocytes into subpopulations enriched for macrophages or mast cells, PAI was found to be principally a product of the macrophage population.

1.1.3. Normal pleural leukocytes show plasminogen activator (PA) activity intracellularly

In contrast to the great production and release of PAI by pleural leukocytes in culture, a predominant intracellular PA activity was demonstrated. The activity was mainly located in mast cells with limited activity in macrophages as confirmed by fibrinolytic activity in cell lysates. Many studies have shown that cell lines release PA and PAI concomitantly, as confirmed by the study of Dong *et al* (1988) that inside tumour cells, PA was the predominant activity that overshadowed PAI. In the extracellular fluid, however, PA activity was masked by powerful PAI. After secretion, PA and PAI tend to form complexes (Philips *et al*, 1984; Vassalli and Dayer, 1984). Due to the formation of enzyme-inhibitor complexes, active enzyme would be rapidly inhibited by the co-secreted ligand, which may explain why neither PA nor PAI activities could be detected in pleural lavage found in the present study (discussed in Section 1.3).

Without doubt PAs play an important role in fibrinolysis. They degrade

fibrin and other extracellular matrix components in the presence of plasminogen and participate in important physiological reactions such as inflammation, malignant invasion, and metastasis (Kruithof *et al*, 1986). The present study, previous work from our laboratory (Donaldson *et al*, 1988_c) and results of other workers (Todd, 1964), have all indicated that mesothelial cells also synthesize a PA. In the pleural cavity, the metabolism of extracellular matrix could be critical in maintaining the normal functions of the pleura, and the balance between PA derived from mast cells, macrophages as well as mesothelial cells and PAI from mainly macrophages is likely to play an important role *in situ*.

1.1.4. Endotoxin and PAI production by normal pleural leukocytes

Many workers have reported the production of PAI by cell lines, but with a requirement for the presence of stimuli. The macrophages lavaged from normal human lungs, for example, do not contain PAI and only express and secrete the inhibitor after stimulation with endotoxin *in vitro* (Chapman and Stone, 1985_b); PAI can also be secreted by casein-elicited macrophages (Saksela *et al*, 1988). The media used in present experiments have been tested for endotoxin levels and results revealed low level contamination of endotoxin in the media up to 10^6 pg/ml. It is presumed that these low levels of endotoxin may be responsible for the spontaneous production of PAI by normal pleural leukocytes.

Nevertheless, the present study shows that when 100 ng/ml of LPS was added into pleural leukocyte culture, there was no considerable enhancement of PAI production by the cells. This result is not consistent with other workers' findings which demonstrated that generation of PAI was in response to endotoxin stimulation (Colucci *et al*, 1985; Crutchley and Conanon, 1986). This might be because normal pleural leukocytes have reached their maximal production of PAI in response to low contamination of endotoxin in the media, and that higher exogenous endotoxin causes no further stimulation. No further attempts were made to discern the role of endotoxin in PAI secretion by pleural leukocytes.

1.2. Fibrinolysis induced by pleural leukocytes from rats intratracheally instilled with asbestos

Exposure to asbestos is associated with diseases of the lung parenchyma and airway (Wagner, 1986; Hirsch and Bignon, 1985). Of particular interest to the present study however, are the pleural pathological responses such as inflammation, fibrous adhesions, fibrosis and neoplasia (Herbert, 1986). One factor that could lead to these problems is altered fibrinolytic activity, since disorder of the fibrinolytic enzyme system could be important in leukocyte activation and altered metabolism of extracellular matrix in the pleural wall. Moreover, matrix

abnormalities are likely to be central to the pathological process. Since the production of appropriate PA/PAI levels by pleural leukocytes could be an important factor in maintenance of the normal integrity and architecture of the pleura.

1.2.1. Pleural leukocyte population after intratracheal instillation (I/T) of crocidolite asbestos

When crocidolite asbestos was intratracheally instilled into rat lungs and different post-exposure time points were selected, there were modest changes to the pleural leukocyte population with significantly increased number of macrophages and eosinophils. The most notable change was the total cell number increasing from 4.22 to 6.69×10^6 . Additionally, in the acute period after instillation of asbestos some PMNs appeared in the pleural space.

Miller *et al* (1989) showed that the number of mature macrophages and macrophage-like cells increased in pleural space, proportionally, in ceramic fibre-exposed rats compared to the control. Oberdoerster *et al* (1983) described, in a rat intratracheal study, peroxidase-positive macrophages (indicative of newly arrived macrophages) showed an increase in pleural space after amosite asbestos exposure and remained high throughout the study. A recruitment of pleural macrophages after fibre deposition in the lung was found in the present study. Since macrophage-derived chemotactic factor was found to play an important role in pulmonary leukocyte recruitment in response to instilled particulates or asbestos fibres (e.g. Hayes *et al*, 1990; Adamson and Bowden, 1982), attempts were made to demonstrate pleural leukocyte-derived chemoattractants for macrophages or PMNs in the present study but no chemoattractants were detected. The results suggest that pleural leukocytes are not responsible for the recruitment of pleural macrophages and PMNs caused by asbestos I/T, although there could be several reasons for failure to demonstrate chemotaxin. Antony *et al* (1989) reported that pleural mesothelial cells stimulated by asbestos *in vitro* released chemotactic activity for PMNs. Although no evidence is available in the present study to show the translocation of asbestos fibre from the airspace to the pleural space (discussed Section 3), it is still possible that pleural mesothelial cells were stimulated by asbestos to produce chemoattractants.

In the later stage of asbestos-induced inflammation, the increase in macrophages may result from division of resident macrophages (Adamson and Bowden, 1982; Sibille and Reynolds, 1990). A study on individuals exposed to asbestos showed that an increased number of alveolar macrophages was related to both monocyte recruitment through lymphocyte-derived chemotactic factor(s) and

to local proliferation of alveolar macrophages (Spurzem *et al*, 1987). The finding of Lemaire (1985_b) also indicated that macrophage replication may contribute to the long increase in inflammatory cells in animal treated with long fibre chrysotile. Intraperitoneal injection of asbestos fibres induced an inflammatory exudate characterized by increased macrophage numbers (Hamilton *et al*, 1976) and in which approximately 70% of the asbestos-induced macrophage incorporate [³H] thymidine indicating that many of the cells are young cells, having recently divided locally. No attempt was made to determine whether the increased leukocyte number found in the present study was due to local macrophage replication in the pleural space.

With increasing time after crocidolite asbestos deposition, the total numbers of pleural eosinophils increased significantly. It is not clear what role eosinophils play in the asbestos-induced events in the pleural space. However, the production of inflammatory mediators or cytokines (discussed in Section 2) was not related to changes in this cell population. Seaton *et al* (1989) suggested that eosinophilic effusion in the pleura caused by fungal and viral infections result from the release of eosinophil chemotactic factor from white cells in blood that have escaped into the pleural cavity. In the present study, recruited PMNs or macrophages in the pleural space may contribute to the increase in eosinophil numbers but this was not investigated.

1.2.2. Effect of I/T asbestos on PAI production by pleural leukocytes

Evidence that exposure to asbestos causes abnormalities of the fibrinolytic system has been presented previously. Cantin *et al* (1989) found that the early lower respiratory tract inflammatory response in sheep resulting from chronic asbestos exposure is associated with a marked increase in PA, both at the alveolar macrophage surface and in the alveolar extracellular milieu. Other studies also showed that exposure of mouse peritoneal macrophages to chrysotile for two hours induced production of PA in macrophages (Lison *et al*, 1989) and intraperitoneal injection of asbestos induced PA secretion by the macrophages in mice (Hamilton *et al*, 1976). The present finding that pleural leukocytes from rats exposed to asbestos express increased PAI in culture contrasts with these observations. In the other studies cited, however, macrophages made direct contact with the asbestos whereas, in the present study, the asbestos fibres have failed to transfer from the lung parenchyma to the pleural space in appreciable numbers, if at all. In the model used in the present study, instillation of asbestos into the lungs therefore may affect the pleural leukocytes by the transfer of molecular signals to the pleural cavity from the adjacent parenchyma.

PAI activity in the pleura increased during pathological changes as demonstrated by Agrenius *et al* (1989) that 6 hr after quinacrine pleural instillation for pleurodesis treatment of malignant pleural effusion, the concentration of PAI-1 increased four fold and that of D-dimer, a product of lysis of the fibrin in pleural fluid was 5 times higher before treatment. Idell *et al* (1989) showed that exudative pleural effusions from patients with congestive heart failure, loculated pleural effusions, and other conditions, are characterized by increased procoagulant and decreased fibrinolytic activity. The present study also suggests that there may be a localised fibrinolytic deficit in the pleura of asbestos-exposed lung.

Crystal *et al* (1981) suggested that activated lung inflammatory leukocytes play a critical role in mediating the pathogenesis of extracellular matrix alterations and fibrosis in asbestos induced interstitial lung diseases. Spregners and Kluft (1987) found that newly recruited macrophages are normally more active in producing inflammatory mediators. As discussed in Section 1.2.1, asbestos I/T causes either recruitment or local division of pleural macrophages. Those "young" macrophages are likely to produce PAI actively so causing inhibition of fibrinolysis although they may also have enhanced connective tissue protease activity (Brown and Donaldson, 1988).

However, the increased PAI production by pleural leukocytes after intratracheal asbestos cannot be explained completely by the recruitment of macrophages since one day after asbestos instillation, the higher dose of asbestos induced markedly enhanced production of PAI by the leukocytes but no substantial recruitment of macrophages had yet appeared. This means that, very likely some inflammatory signal travels from asbestos-exposed parenchyma to the pleural space. The non-specific airspace irritation alone is not, however, the cause of the response of the pleural leukocytes since saline-instilled group gave no difference with controls.

In the present study, pleural leukocytes appear to respond to signals from lungs inflamed by asbestos deposition. A strong pleural inflammatory reaction characterized by increased PAI production is then evoked as soon as these inflammatory signals reach the pleural space and this reaction is related to the intensity of inflammation and so, very likely, to the strength of the signals.

It is reported that neither fibrin nor fibrinogen are normal constituents of either the pulmonary interstitium or the alveolar space. However, fibrin deposition is a prominent component of a number of acute and chronic inflammatory disorders of the lungs. The sites of early fibrin deposition correlate with the

location of the subsequent fibrotic process, suggesting that fibrin has importance for fibroblast attraction, attachment, spread, growth and proliferation (Chapman and Stone, 1985_b; Chapman *et al*, 1986). Because fibrin resorption is virtually dependent on synthesis and expression of a classic plasminogen activator of the urokinase type (Chapman and Stone, 1985_b), the increased PAI may lead directly to increased deposition of fibrin and other extracellular matrix components, such as fibronectin. These may then form a scaffold for fibroblast adherence and proliferation to occur.

Hopper *et al* (1981) have demonstrated the deposition of fibrin on the surface of peritoneal macrophages *in vivo*. In another study, based on their findings, they proposed that the lymphokine-activated macrophages have thromboplastic properties which would enable local activation of the extrinsic clotting pathway (Geczy and Hopper, 1981). Because macrophages can produce fibrin or fibrinogen on their own surface, although there was no severe pleural inflammation, pleural macrophages could serve as a nidus for pleural fibrosis by forming the fibrin scaffold around themselves. In the pleural cavity, deposition of fibrin at the surface of mesothelial cells and fibroblasts could also activate fibroblast proliferation and facilitate the production of fibrous adhesions between the two opposite layers of the serosal membrane (Bignon and Gee, 1985). Ferro *et al* (1989) reported that macrophages activated by fibrin increase albumin permeability across pulmonary artery endothelial monolayers, which is mediated by an oxidant-dependent mechanism. The deposition of fibrin may cause increased leakage which may result in positive feedback of inflammation.

The relationship between deposition of fibrin and fibrosis in the pleural cavity is indirectly demonstrated by the fact that intrapleural urokinase inhibited the development of fibrosis in the rabbit pleural spaces inoculated with high-dose tetracycline (Good *et al*, 1979). This implies that enhanced pleural fluid clotting and inhibition of fibrinolysis is important for inducing pleural fibrosis. The present observations showing increased PAI production by pleural leukocytes during asbestos-induced pleural injury, reveals again that asbestos related pleural fibrosis may be initiated by the inhibition of fibrinolysis.

It has been shown that mesothelial cells have an important role in fibrinolysis (Porter *et al*, 1971), and this is supported by the present study. Whitaker *et al* (1982_a) found that the fibrinolytic activity of mesothelial cells is depressed by damage to the cells. Donaldson *et al* (1988_c) demonstrated that crocidolite asbestos inhibited plasminogen dependent fibrinolysis induced by mesothelial cells. However, in the *in vivo* situation, mesothelial cell-mediated

fibrinolysis may be inhibited by PAI released by pleural leukocytes after airspace asbestos exposure as shown here. Persistent depression of fibrinolysis will, therefore, be associated with the development of pleural fibrosis (Buckman *et al*, 1976) through colonisation of fibrin by macrophages and fibroblasts under the particular conditions pertaining in the pleural space with asbestos exposure (Donaldson *et al*, 1988_c).

The highest dose of instilled asbestos caused recruitment of PMN, a cell type not normally present in the pleural leukocyte population. Macrophages may cause PMN accumulation in the pleural space (Schoenberger *et al*, 1982). However, there was no correlation between PMNs and PAI production in this study. The role that PMNs play in asbestos-induced pleural inflammation remains unclear. Similarly, the role of eosinophils in the inflammatory events occurring in the pleural cavity of asbestos-exposed rat is not further elucidated in the present study. The release of mediators from human mast cells is one of the central events in inflammatory reactions involving the human lung (Marone, 1985). Mast cells are also considered to play a role in the development of fibrotic responses (Miller *et al*, 1989). However, the present study shows no evidence that mast cells are involved in PAI production by pleural leukocytes. The functions of the mast cells in the events leading to asbestos related pleural pathology therefore remains to be investigated.

1.2.3. Effect of I/T asbestos on pleural leukocyte intracellular PA activity

Cantin *et al* (1989) have shown evidence that chronic exposure to asbestos caused a markedly increased PA activity in both alveolar macrophage surface and alveolar extracellular milieu. The study of Donaldson *et al* (1987) indicated that plasminogen-dependent fibrinolysis induced by alveolar macrophages was inhibited by chrysotile asbestos *in vitro*. The altered PAI levels in pleural leukocyte supernatants from rats exposed to asbestos may result from either increased PAI activity or decreased PA activity. Therefore, intracellular PA activity of pleural leukocytes has been determined. PA activity in the lysates of control pleural leukocytes and pleural leukocytes from rats following 10 mg crocidolite asbestos I/T showed no significant difference. This confirmed apparently that the increased PAI activity is indeed specifically caused by increased PAI production rather than decreased PA activity.

1.3. PA and PAI activity of pleural lavage

The study of Chapman and Stone (1985_b) indicated that human alveolar fluid from all normal volunteers contained PA whereas no PAI activity was observed. In contrast, alveolar fluid from patients with sarcoidosis and idiopathic

pulmonary fibrosis had low or absent PA activity (Chapman *et al*, 1986). Idell *et al* (1988) found that in control bronchoalveolar lavage (BAL) samples, there was relatively low levels of procoagulant activity and relatively high levels of fibrinolytic activity. After induction of lung injury by oleic acid, the procoagulant activity of BAL was markedly increased whereas fibrinolytic activity was either depressed or undetectable. However, the work of Sitrin *et al* (1987) showed that normal BAL fluid contained high levels of procoagulant activity which did not vary after the onset of acute lung injury induced in rabbits by an intravenous injection of phorbol myristate acetate (PMA). There were low levels of PA activity in normal BAL fluid and the activity increased 9.3-fold over control values by 12 hr after PMA injection. These studies indicate that, during acute inflammation or pulmonary diseases, fibrinolytic activity in BAL is altered.

The present study confirmed that there was no detectable fibrinolytic activity in normal pleural lavage. This could be due to an equal balance between secretion of PA and PAI by pleural leukocytes with formation of PA and PAI complex (Dong *et al*, 1988). An alternative explanation is that there is no active production of PA or PAI under the unstimulated situation in the pleura. Fibrinolytic activity in pleural lavage during injury caused by asbestos or other agents needs further investigation.

1.4. Fibrinolytic activity of pleural leukocytes after I/T *C.parvum*

1.4.1. Pleural leukocyte components after I/T *C.parvum*

Instillation of *C.parvum* induced severe alveolar inflammation, characterized by recruitment of large numbers of PMN. Although, during acute inflammation, the total cell number (mostly PMN) was about 6 times higher than normal, the neutrophil alveolitis lasted only 3 days whilst the macrophage alveolitis was a relatively long-lived process. The same inflammatory pattern was found in the pleural space after I/T *C.parvum* with transient pleural PMN inflammation and long standing macrophage pleuritis. In our study, the proportions of pleural PMN increased from 0 to 33% of total leukocytes, then rapidly decreased by day 5, whereas the macrophage pleuritis persisted. The very similar inflammatory patterns in both alveolar and pleural spaces indicated that there may be some communication of inflammatory signals between the two compartments.

C.parvum-elicited bronchoalveolar inflammation has been investigated by Donaldson *et al* (1988_b) and Brown and Donaldson (1988). As demonstrated by many workers, macrophage-derived chemoattractants may be responsible for the alveolitis. Adamson and Bowden (1982) suggested that particulate instillation in the lung stimulates the generation of a factor(s) chemotactic for PMNs; mononuclear

cells are also attracted to the alveoli. Hayes *et al* (1990) also suggested that alveolar macrophages may release a PMN chemotactic factor responsible for the recruitment of PMN into the alveolar space.

The chemotactic activity for PMN and macrophages of pleural leukocyte supernatants from rats with *C.parvum*-induced inflammation was investigated. The supernatant of pleural leukocytes from rats 5 days after *C.parvum* instillation showed some chemotactic activity for macrophages *in vitro*, whereas it showed slight inhibition of PMN chemotaxis. As reported by Donaldson *et al* (1989_b), *C.parvum*-elicited alveolar neutrophil supernatants caused inhibition of chemotaxis for macrophages. Antony *et al* (1989) reported that asbestos *in vitro* stimulated mesothelial cells to release a chemoattractant for PMN, so it is presumed that pleural mesothelial cells stimulated by mediators from adjacent *C.parvum*-induced inflammatory lung may be responsible for the chemotactic activity for PMN recruitment to the pleura, this was not, however further investigated in the present study. The recruitment of PMN ended very rapidly and this may reflect the defensive functions of the pleura in restricting further inflammation as well as reflecting the removal of the inflammagen from the lung. The increased macrophage chemoattractive activity in pleural leukocyte supernatants by day 5 may be responsible for the continuous recruitment of macrophages into the pleural space after I/T *C.parvum*.

1.4.2. PAI production by pleural leukocytes during inflammation caused by I/T *C.parvum*

The present study found that PAI activity by pleural leukocytes was significantly increased after I/T *C.parvum*. However, this returned to normal levels by day 5. Sibille *et al* (1990) suggested that there is interaction between alveolar macrophages and PMN in the lung. Upon stimulation by various stimuli, alveolar macrophages release chemotactic factors for PMN. This factor will attract PMN from the capillary lumen to the alveolar space. Once in the alveolar lumen, the PMN can be activated by various factors, including alveolar macrophage-derived monokines and release active products, such as oxidants, bioactive lipids and proteases, which are likely to influence the function of alveolar macrophages and other fixed lung cells. Presumably, during neutrophil pleuritis, the recruited PMN may influence pleural macrophage production of PAI. The similarity in the change in pleural PMN numbers and PAI production during the study period supports this hypothesis. Since Kopitar *et al* (1985) have isolated urokinase type PAI from cytosol of human peripheral PMN, newly recruited PMN may partly contribute to the increased PAI activity by pleural leukocytes.

Locally reduced fibrinolysis could promote alveolar fibrin deposition which may influence tissue repair (Section 1.2.2). In pleural effusion, a strong inflammatory reaction with a swollen pleural wall and fibrin deposition is sometimes seen (Widstrom *et al*, 1982). The rapid return of the PAI levels to normal in the present study supports the observation that pleural fibrosis rarely results from acute, resolving pneumonitis.

2. Cytokine production by pleural leukocytes and the effect of intratracheally instilled asbestos

2.1. Interleukin-1 (IL-1) elaboration by pleural leukocytes

2.1.1. Normal pleural leukocytes produce IL-1 in culture

Although a large body of work has been done to study IL-1 production by monocyte/macrophage (Martin and Resch, 1988), almost no attention has been given to pleural leukocytes in this respect. In view of the important role of the pleura in basic lung functions and the pathology caused in the pleura by fibrous dusts, the production of IL-1 by pleural leukocytes was determined.

The present study indicated that normal unstimulated pleural leukocytes secrete significant IL-1 *in vitro* and this secretion can be enhanced further by stimulation with 100 ng/ml LPS. By contrast, studies on IL-1 production by human monocytes reveals no intracellular or extracellular IL-1 production by unstimulated monocytes (Dinarello, 1984) and most normal cell types and a number of cell lines produce IL-1 only in response to a variety of stimulants (Oppenheim *et al*, 1986). Constitutive production of IL-1 by normal cells can probably be attributed to a continuing response to prior stimulation *in vivo* or alternatively, to the presence of endotoxin contamination of tissue culture media or plastics. It was shown that culture media, blood separation media, fetal calf serum, and organic buffer used in culturing IL-1 producing cells may contain endotoxin, depending on the source. The "spontaneous" activation of IL-1 secretion by cells may also result from procedures of harvesting, washing, and cell adherence (Dinarello, 1984). The measurement of endotoxin in media and serum used in the present study was carried out and low levels of endotoxin contamination, ranging from 0-108 pg/ml, were found. It is possible therefore that the spontaneous production of IL-1 by pleural leukocytes in culture is the result of stimulation of the cells with low levels of endotoxin contamination in the media. However, as a serosal cell population, similar to pleural mesothelial cells which are highly susceptible to damage from substances that they do not normally encounter (Herbert, 1986), it is also possible that pleural leukocytes may be highly susceptible to alteration in their environments and the effect of this could be

stimulation of IL-1 production.

Although normal pleural leukocytes produced relatively high IL-1 activity in culture, these cells were still able to produce significantly more IL-1 activity in response to 100 ng/ml LPS stimulation. This suggests that, if LPS was responsible then, it was at sub-optimal levels. The fact that alveolar leukocytes produced much less IL-1 "spontaneously" than did pleural leukocytes also suggests that either pleural leukocytes do indeed produce truly spontaneous IL-1 or that alveolar leukocytes are less sensitive to the low level of endotoxin present in the media.

2.1.2. Pleural lavage fluid inhibits IL-1 activity in the pleural leukocyte supernatants

In the present study there was no detectable IL-1 activity in normal pleural lavage fluid. In fact, pleural lavage fluid exhibited an inhibitory effect on the activity of both the IL-1 β standard and pleural leukocyte IL-1 in a concentration related manner. This result suggests that there is an IL-1 inhibitor in normal pleural lavage fluid.

Many studies have demonstrated specific IL-1 inhibitors from different cellular sources. The work presented by Gosset *et al* (1988) indicated that asthmatic alveolar macrophage supernatants contained no detectable IL-1-like activity after stimulation by allergen or anti-IgE. In contrast, an IL-1 inhibitory factor, released by alveolar macrophages, was demonstrated in the supernatants. An alveolar macrophage-derived IL-1 inhibitor was also confirmed by Rochemonteix *et al* (1990). Human monocyte-derived IL-1 inhibitors have also been demonstrated by Arend *et al* (1989) and Schur *et al* (1990). Mechanisms of action of IL-1 inhibitors has been proposed as: 1) action on IL-1 receptors to prevent thymocyte proliferation (Schur *et al*, 1990), 2) action at a post-receptor stage after the initial activation of the cell by IL-1 (Brown and Rosenstreich, 1987).

IL-1 inhibitor(s) may play an important role in modulating the effects of IL-1 in the microenvironment. Rochemonteix *et al* (1990) suggested that the production of a specific IL-1 inhibitor by alveolar macrophages may be of importance in protecting the alveolar environment from the deleterious effects of excessive IL-1 production. In particular, in pleural space, the regulation of IL-1 activity by its inhibitor would be anticipated to have very important role in maintaining pleural functions.

There could be therefore two possible explanations for the failure to detect IL-1 activity in pleural lavage fluid, 1) there is a balance between the secretion

of IL-1 and IL-1 inhibitor, 2) the pleural leukocytes *in vivo* are much less stimulated than when they are cultured, because of the culture conditions. There was no attempt to characterize the IL-1 inhibitor found in the present study as this was outside the scope of the study but should be an important target for future studies.

2.1.3. IL-1 production by pleural leukocytes after intratracheal crocidolite asbestos

In the present study, it is shown that up to 14 days post intratracheal instillation of 5 mg crocidolite, lavaged pleural leukocytes produced significantly less IL-1 in culture than control. However, one month after instillation, this production increased dramatically and was above that of controls. The change of IL-1 production by pleural leukocytes at various times post asbestos injection cannot be simply explain by alterations in cell populations. It is presumed therefore that during asbestos exposure, the capability of pleural leukocytes to produce IL-1 may be impaired and so that less IL-1 activity can be measured.

However, the change in IL-1 activity shown by pleural leukocytes may be also influenced by the presence of IL-1 inhibitor. Specific IL-1 inhibitors have been described by many workers (Arend *et al*, 1989; Schur *et al*, 1990; Gosset *et al*, 1988). It has been noted that IL-1 inhibitors are concurrently produced by the cells releasing IL-1 (Tiku *et al*, 1986_b; Larrick, 1989). Since we demonstrated the activity of IL-1 inhibitor in pleural lavage (Section 2.1.2), presumably, there may be secretion of IL-1 inhibitor by the cells elaborating IL-1 concurrently or by other cells in the population so causing decrease of IL-1 activity. Gosset *et al* (1988) showed that control alveolar macrophages released IL-1, but that, after stimulation with IgE, the cells switch to production of an IL-1 inhibitor. Because the release of IL-1 or its inhibitor appears to be dependent on the state of cell activation, asbestos deposited in the nearby airspaces could produce a signal that triggered pleural leukocytes to release IL-1 inhibitor.

It has been reported that normal alveolar macrophages are less sensitive to LPS than blood monocytes and they release significantly less IL-1 than blood monocytes (Wewers *et al*, 1984). In addition, denser alveolar macrophages (less mature, more monocytic) elaborate more IL-1 than less dense alveolar macrophages (Haq and Maca, 1986; Fuchs *et al*, 1988). Since young mononuclear phagocytes are the prime source of IL-1, IL-1 release in the pleura may be regulated, at least in part, by factors that control the influx of young monocytes (Wewers *et al*, 1984). In the present study, asbestos-recruited leukocytes which are possibly more monocytic could produce increased IL-1 which could overwhelm the

inhibitor levels.

Despite the fact that increasing the dose of injected crocidolite resulted in only minimal changes in pleural cell population, with the appearance of a low level of PMNs in the pleural cavity, an apparent asbestos dose-dependent decrease in IL-1 production by the leukocytes was observed at one day post instillation. Since we have shown there is no substantive translocation of asbestos fibre from the lung to the pleura in the present model (Section 3), then this is likely to be the result of an inflammatory signal travelling from the lungs to the pleura. The response to this signal would be decreased production of IL-1 or increased production of IL-1 inhibitor.

It has been demonstrated that asbestos exposure lead to increased IL-1 release by alveolar leukocytes: Kagan *et al* (1985_a) showed that inhalation of either crocidolite or chrysotile asbestos in the rat was associated with augmented release of IL-1 by alveolar macrophages. *In vitro* exposure of peritoneal macrophages to chrysotile also resulted in increased intracellular levels of IL-1 in those cells (Godelaine and Beaufay, 1989). However, suppression of IL-1 release was found with pleural leukocytes in the present study after asbestos exposure. It is possible that the presence of asbestos-induced alveolar inflammation results in a suppressive pleural response in order to preserve normal lung functions. Since we found the same result with another cytokine, TNF, and with IL-1 using another inflammogen, *C.parvum* (Section 2.1.4), it seems that this short term suppression may have substantial beneficial value in the pleural space. However, in the longer term, the IL-1 production was increased. IL-1 may stimulate fibroblasts and their functions such as collagen synthesis, prostaglandin production and fibroblast migration (Postlethwaite *et al*). Thus, the long term increase in IL-1 production could lead to fibroplasia in the pleura. Increased production of PAI by pleural leukocytes, which has been demonstrated in the same model, could produce a fibrous matrix for fibroblast growth and also encourage fibrous tissue growth.

As reported by Pozo *et al* (1990), LPS-stimulated mouse eosinophils were able to express IL-1 mRNA in their cytoplasm and produce IL-1 activity; it was however less than in that of mouse macrophages. Since eosinophils accounted for a low but significant percentage (6%) of the normal pleural leukocytes, their role in production of IL-1 by pleural leukocytes should be considered. However, in the present study, there was no relationship between the number of eosinophils and IL-1 activity.

2.1.4. IL-1 production by pleural leukocytes is decreased during pleural inflammation induced by I/T *C.parvum*

As a low molecular weight factor which mediates a wide variety of pro-inflammatory effects, IL-1 has been confirmed to be increased in inflammation (Lassle *et al*, 1990; Fuchs *et al*, 1988). However, the present study indicated that during *C.parvum*- induced pleural inflammation, IL-1 production by pleural leukocytes was decreased compared to controls and a greater reduction of IL-1 production was observed at day⁵ than day¹ after *C.parvum* I/T. It is possible that the initial reduction in IL-1 activity may be due to the accumulation of PMN in the pleural space since 1) the percentage of macrophages was decreased by the presence of neutrophils; 2) IL-1 inhibitor may be constitutively present in normal neutrophils (Tiku *et al*, 1986b). During the later stages after *C.parvum* instillation, the further reduction in IL-1 production by pleural leukocytes might result from the additional secretion of IL-1 inhibitor by newly recruited macrophages (Section 1.4.1). There are reports of IL-1 inhibitors produced by normal human monocytes (Arend *et al*, 1989; Schur *et al*, 1990) as well as human alveolar macrophages (Gosset *et al*, 1988) during inflammatory and immune response. The present findings were consistent with a part of the present study (Section 1.3) which showed a decreased response in IL-1 production by pleural leukocytes after deposition of asbestos in airspaces.

2.1.5. The effect of asbestos in combination with other dusts *in vivo* on IL-1 production by pleural leukocytes

2.1.5.1. Pleural leukocyte components one month after intratracheal instillation of asbestos in combination with other dusts

The present study showed that the deposition of crocidolite asbestos in combination with other dusts in the lungs caused considerable recruitment of pleural leukocytes in which eosinophils increased most significantly. The results reveal once more that eosinophils may play some role in asbestos-induced pleural pathology in this model. However, there was no direct relationship between eosinophils and any inflammatory parameter in present study. Eosinophils are considered to affect the functions of mast cells, fibroblasts and mesothelial cells (Venge, 1990; Bignon and Gee, 1985). Eosinophils associated with, or are predominant in other pleural inflammatory diseases (Bignon and Gee, 1985). In view of these points, further investigation on the role of eosinophils in pleural pathological change due to asbestos is necessary.

2.1.5.2. The effect of asbestos in combination with other dusts *in vivo* on IL-1 production by pleural leukocytes

The present study showed that one month after intratracheal instillation, only quartz caused significantly increased IL-1 production by pleural leukocytes.

This may be related to its ability to penetrate to the pleural space (Davis *et al*, 1991). Crocidolite asbestos did not cause increased IL-1 release. The explanation of this may be that the dose of asbestos (2.5 mg) used in the study is not high enough to show stimulatory effect. Nevertheless, there were no important effect in stimulating IL-1 production caused by dusts in combination.

2.2. Tumour necrosis factor (TNF) production by pleural leukocytes

2.2.1. Production of TNF by normal pleural leukocytes in culture and the effect of LPS stimulation

Mononuclear phagocytes are a major source of the cytokine, TNF, and TNF production by the cells can be increased by exposure to LPS *in vitro*. Resting alveolar macrophages do not express the TNF mRNA transcripts but, when activated by LPS, they expressed TNF transcripts, synthesized and released TNF (Martinet *et al*, 1988). Rich *et al* (1989) and Nissen-Meyer *et al* (1988) found that the production of TNF by LPS-induced alveolar macrophages and monocytes was as much as 10 and 100-fold greater than that by non-induced cells respectively. Adams and Czubrynski (1990) also demonstrated that bovine monocytes release TNF α in response to stimulation with LPS and this release peaks at 1-3 hr post LPS treatment, with subsequent decline to background levels by 18 hr. The present study confirmed that there were very low levels of endotoxin (0-108 pg/ml) in the media used in the experiments. A contribution of this low level of endotoxin contamination to the "spontaneous" production of TNF by pleural leukocytes in culture cannot be ruled out although the depletion of endotoxin in the media by polymixin B had no influence on spontaneous TNF release. However, despite this "spontaneous" secretion of TNF, pleural leukocytes could still be stimulated with 100 ng/ml LPS in culture to release markedly higher TNF activity.

TNF anti-serum was used in the study and the results confirmed the specificity of the assay for leukocyte TNF.

2.2.2. Pleural lavage inhibits TNF activity in pleural leukocyte supernatants

In the present study, it was found that there was no detectable TNF activity in the pleural fluid. In fact, pleural lavage fluid inhibited TNF activity in both the pleural leukocyte supernatants and the TNF α standard. The fact that there was no TNF in pleural lavage but pleural leukocytes released TNF in supernatants in culture could be due to several factors. The most likely of these is the presence of functional inhibitor of TNF in the lavage fluids tested. Giovine *et al* (1988) demonstrated the co-existence of TNF and its inhibitor in synovial fluids from rheumatic patients. Lin *et al* (1988) showed that spleen cells from

normal, untreated mice produce a factor that inhibits TNF cytolytic activity and the factor acts on the targets rendering them refractory to TNF binding. Seckinger *et al* (1989) have purified an inhibitor of TNF α from urine and characterized its mechanism of action as the interference of the interaction of the ligand and its receptor. We suggest that the TNF inhibitor found in pleural lavage may play an important role in controlling inflammatory reactions in the pleural space which are mediated by TNF.

2.2.3. The effect of asbestos in the airspaces on TNF production by pleural leukocytes

Normally, processes of inflammation and response to injury are associated with augmented release of TNF (Larrick and Kunkel, 1988). Interestingly, our results demonstrate that deposition of asbestos in the airspace causes pleural leukocytes to secrete significantly less TNF than control pleural leukocytes. In a study involving intrapleural injection of crocidolite asbestos (Edwards *et al*, 1984), lysosomal enzymes and non-specific esterase were described in mononuclear cells and giant cells in asbestos-induced granulomas. In the present model, following I/T asbestos, there is no substantial transfer of asbestos fibres to the pleural cavity (Section 3) and we therefore suggest that some diffusible mediator(s) may play a role in spreading inflammation from the airspace, where asbestos deposits, to the pleural space.

We have demonstrated that pleural lavage fluid contains the TNF inhibitor activity. In the present study, it was found that pleural mesothelial cells are not responsible for the TNF inhibitory effect, so it is likely that an inhibitor is produced by pleural leukocytes which also are the source of TNF. The asbestos-induced reduction of TNF activity in pleural leukocyte supernatants found in the present study may be explained by the co-release of TNF inhibitor. The normal situation is likely to depend on the balance of activities of TNF and its inhibitor. For normal pleural leukocytes in culture, the production of TNF is predominant. After asbestos I/T, inflammatory mediators from the lung may trigger the expression of the TNF inhibitor gene or the release of the inhibitor, and lead to the decreased TNF activity seen in culture. On the other hand, the mediators may block the expression of the TNF gene or the release of TNF, which could also result in a predominance of the TNF inhibitor in the culture.

Rich *et al* (1989) and Okubo *et al* (1990) demonstrated that the capability of alveolar macrophages to produce TNF was significantly greater than that of blood monocytes. In addition, *in vitro* maturation of blood monocytes resulted in an increased production of TNF. The present study demonstrated recruitment of

pleural macrophages after intratracheal asbestos. These newly recruited macrophages (monocytes) are therefore likely to produce less TNF and this may explain the persistent reduction of TNF activity in the longer term after asbestos exposure.

TNF has been shown to play an important part in silica-induced pulmonary fibrosis in mice (Piguet *et al*, 1990_a) and possibly to have a role in coal workers' pneumoconiosis (Borm *et al*, 1988). In the present study, TNF production by pleural leukocytes decreased as the result of intratracheal asbestos and this reduction was related to the dose of asbestos deposited in the airspaces. Presumably, as an inflammatory mediator, TNF functions beneficially for the body during inflammation. The suppression of TNF activity described here may prevent second order responses of the pleura to asbestos deposition in the lung. A persistent deficit in these TNF-mediated defensive responses, which could include recruitment of leukocytes and their activation for inflammation and immune defence, could play a role in subsequent pleural pathology.

Although it was suggested that mast cells may play a role in secretion of TNF (Beutler and Cerami, 1988_a), there was no circumstantial evidence from mast cell numbers that they were the source of TNF. However, since mast cells have been suggested to play a role in the response to fibres (Miller *et al*, 1989), their functions in the pleural space require further investigation.

2.2.4. Effect of *C.parvum*-induced pleural inflammation on the TNF activity produced by pleural leukocytes

At 1 day post I/T *C.parvum*, it was evident that pleural leukocytes produced significantly less TNF activity and this reduction was even greater by 5 days. During *C.parvum*-induced pleuritis, the most marked changes in cell components were:- (1) considerable increase in PMN numbers on day 1 with rapid recovery to normal, (2) significant and persistent enhancement of macrophage numbers.

TNF-specific inhibitors have been confirmed by Giovine *et al* (1988) and Seckinger *et al* (1989). The present study also indicated that there is a TNF inhibitor in pleural lavage fluid. The decreased TNF activity after I/T *C.parvum* may be explained by increased production of TNF inhibitor by pleural leukocytes (Section 2.2.3) since there is dramatic recruitment of the leukocytes after I/T *C.parvum*. However, it seems also possible that decreased TNF activity was caused by the altered pleural leukocyte components. Studies by Rich *et al* (1989) and Okubo *et al* (1990) revealed that TNF production by alveolar macrophages was significantly more than that by blood monocytes in response to LPS. *In vitro*

maturation of blood monocytes resulted in increased expression of TNF. This suggests that maturation of mononuclear phagocytes leads to a phenotype capable of producing higher levels of TNF. Thus, during *C.parvum*-induced pleural inflammation, the huge PMN number means that there are proportionally less macrophages and also that newly recruited, more monocytic, macrophages would have less TNF-producing capacity than mature macrophages. The decreased TNF activity may result from the recruitment of PMNs in first instance, then from the recruitment of blood monocytes to the pleural space.

2.2.5. Effect of I/T asbestos in combination with other dust on TNF production by pleural leukocytes

In most studies, animals or cells are exposed to single dust types or asbestos fibres for ease of interpretation of results. However, many people are likely to have mixed exposure to different dusts in the workplace. It is important therefore to know the effect of dust combinations on cell functions. To examine this, crocidolite asbestos and other particles, individually and in combination, were instilled and the effect on TNF production by pleural leukocytes was investigated.

One month after intratracheal instillation, asbestos and other dusts alone caused decreased TNF release by pleural leukocytes. It is difficult to understand why TiO_2 , an inert dust, also caused decreased TNF production. However, the crocidolite-induced reduction of TNF release was consistent with observation in earlier part of the study that one month after I/T crocidolite, the TNF production by pleural leukocytes was dramatically decreased (Section 2.2.3). However, any combination of asbestos with particles caused dramatic increases in TNF production by pleural leukocytes. The combination of crocidolite and quartz induced up to 4-fold more TNF activity than the control level. Even the combination of crocidolite and TiO_2 could stimulate pleural leukocytes to produce twice the control level of TNF. The results revealed an apparently synergistic interaction between crocidolite fibre and quartz or TiO_2 , in stimulating TNF production by pleural leukocytes.

In experimental studies in rats on the effects of asbestos coupled with the inhalation of TiO_2 or quartz, Davis *et al* (1988_b; 1991) demonstrated that both quartz and TiO_2 together with asbestos caused an increase in the numbers of mesotheliomas compared to asbestos alone. Tumours in animals treated with asbestos plus quartz also tended to occur earlier than tumours with asbestos alone. This was supported by the observation of high mesothelioma incidence (10/328) in pipe makers who had mixed exposure to both crocidolite and "silica" in cement (Finkelstein, 1983). A significant increase in TNF production by pleural

leukocytes after I/T crocidolite plus TiO_2 or crocidolite plus quartz observed in the present study, indicates that mixed exposure to asbestos and particulate dusts may lead to transport of asbestos fibres to the pleural space. An increased transport of particles under such conditions was suggested by Davis *et al* (1991), and may have contributed to the dramatically increased response in pleural leukocyte TNF production. However, the present study failed to demonstrate translocation from the lung to the pleura when asbestos or quartz was instilled alone (Section 3).

It was suggested by Davis *et al* (1988_b) that particulate dusts, especially quartz, have a much greater ability than asbestos fibres to penetrate through the pleura. In the presence of penetrating particle, it was suggested that fibre penetration could be made easier since in animals treated with mixtures of asbestos plus quartz, there was evidence of fibres on the visceral pleural surface. No experiment has yet been carried out to investigate whether asbestos reaches the pleural space in increased amount with the type of dust exposure discussed here. However, in the light of the present findings, that there is a high TNF production by pleural leukocytes after deposition of asbestos plus other dusts, it would be very informative to investigate asbestos translocation to the pleura with such treatments.

2.3. Cytokine production by pleural leukocytes treated with dusts *in vitro*

Although a variety of studies have demonstrated considerable difference in cytokine production by alveolar leukocytes *in vitro* after treatment with different dusts (Kusaka *et al*, 1990_a; Driscoll *et al*, 1990₁), almost no *in vitro* work has been carried out on pleural leukocytes. In the present study, the interaction between dusts and pleural leukocytes was investigated in terms of TNF and IL-1 production. The results indicated that crocidolite, quartz and TiO_2 can stimulate increased production of TNF or IL-1 by pleural leukocytes *in vitro*. However, there was no significant difference between these dusts in ability to cause cytokine production. This indicates that the effect on pleural leukocyte cytokine secretion, of intratracheal asbestos is not simply caused by direct interaction between dust and cells. Some inflammatory signal(s) from adjacent asbestos-inflamed lungs may also mediate the altered pleural leukocyte response.

2.4. The production of TNF and IL-1 by separated normal pleural leukocytes

To investigate possible cellular source of cytokines released by pleural leukocytes, two subpopulations of pleural leukocytes were obtained using separation techniques: a macrophage-enriched population (93% macrophages) and a mast cell-enriched population (76% mast cells and 20% macrophages). TNF and IL-1

activities produced by these two subpopulations were tested and results revealed that both subpopulations secreted less TNF and IL-1 activity in culture than the unseparated populations. This was likely to be caused by slight cellular injury during separation procedure. However, the mast cell-enriched population produced significantly higher activity of both TNF and IL-1 than macrophage-enriched population.

Mononuclear phagocytes such as peripheral blood monocytes (Elias *et al*, 1985; Gerrard *et al*, 1987) and alveolar macrophages (Wewers *et al*, 1984; Haq and Maca, 1986) are reported to be the major cell sources of IL-1. Other cells, however, may also produce IL-1, such as PMN (Canning and Neil, 1989; Tikku *et al*, 1986_a), epithelial cells (Dinarelli, 1984) and eosinophils (Pozo *et al*, 1990). However, no report is known to us that demonstrates mast cells as a cell source of IL-1.

The macrophage (Becker *et al*, 1989; Martinet *et al*, 1988) as well as blood monocytes (Rich *et al*, 1989; Lonnemann *et al*, 1989) are also the major source of TNF production in response to LPS. Several other cell types have also been reported to produce TNF, including T-lymphocytes and smooth muscle cells (Beutler, 1988_a) as well as mast cells. Steffen *et al* (1989) showed the presence of TNF mRNA in the cytoplasm and TNF protein in the granules of individual human mast cells. Mast cells as a source of TNF was also confirmed by Gordon and Galli (1990). Young *et al* (1987) have also demonstrated the presence of TNF in murine mast cells.

It is difficult to explain our findings of increased TNF and IL-1 by the mast cell-enriched population compared to the macrophage-enriched population since intratracheal asbestos-induced changes of TNF and IL-1 activities were not related to mast cell population in the present study. It is presumed that the release of mast cell metachromatic granules which contain pharmacologically potent mediators (Lee *et al*, 1985) might have an important role in stimulating the production of TNF and IL-1 by macrophages. Further research is required but was beyond the scope of the present study.

3. Transfer of particles into the pleural space

Pathological responses have been found in the pleural cavity of asbestos-exposed individuals. These include pleural adhesions, diffuse fibrosing pleuritis and pleural fibrosis as well as mesothelioma (Herbert, 1986; Wagner, 1986). In cytologic studies, it was demonstrated that intrabronchially instilled amosite asbestos had an effect on lavagable pleural cells (Oberdoerster *et al*, 1983).

In view of these findings combined with the present investigation showing functional changes of pleural leukocytes following asbestos deposition in the lung, it is important to know whether asbestos deposited in the lung reaches the pleural space.

3.1. Fate of intratracheally instilled beads

3.1.1. Fate of beads instilled along with asbestos fibres

Small (0.77 μm) fluorescent microspheres (beads) were used as markers of pleural permeability to particles, since they are easily seen when viewed by UV illumination. Results showed that whenever beads alone, or coupled with quartz or chrysotile asbestos, were intratracheally instilled, they were not seen in pleural leukocytes. There were, however, a high percentage of alveolar cells containing beads. Even one month after I/T beads alone or coupled with other dusts, there were no detectable beads in pleural macrophages. These findings indicated that, to all intent and purposes, no beads of this size or shape deposited in the alveolar space transfer into the pleural space. Neither direct penetration, as confirmed by the one day study, nor translocation to the pleura via the lymphatic or blood circulation systems as confirmed by the one month study was evident. In addition, when more severe inflammation was caused in the alveolar spaces by coupled injection of beads with quartz or asbestos and by *C.parvum*, no penetration of particles to the pleura were observed. This reveals that beads of this size in the airspace do not reach the pleura and co-instillation of quartz or asbestos does not assist in this translocation, although Davis *et al* (1991) suggested that quartz may help asbestos fibres to penetrate through the pleural barrier.

A accidental experiment may supply some extra information with regard to fibre transfer. After a rat was intrapleurally injected with beads and chrysotile asbestos accidentally, the pleural leukocyte pattern became very different to that of the normal pattern, with dramatically high total leukocyte number and a high percentage of PMNs. There was also, as would be anticipated, a fairly high percentage of pleural macrophages and PMNs containing beads. This shows that, if beads were transferred to the pleural space they would be indeed phagocytosed by

leukocytes and be detected.

3.1.2. Fate of I/T beads in combination with *C.parvum*

The present observation showing very similar inflammatory patterns in alveolar and pleural spaces indicates the existence of some communication of inflammatory mediators between the two spaces. To test the possibility that particles might be translocated from the lung to the pleural space during *C.parvum*-induced alveolar inflammation, counts of beads within cells in both spaces was carried out. The results indicated that, following deposition in the airspace along with *C.parvum*, although alveolar macrophages and PMNs contain substantial numbers of beads, almost no macrophages or PMNs were found to contain beads at the same time in the pleural space.

The present findings failed to demonstrate beads in the pleural space after intratracheal instillation in various conditions. These results are consistent with the study of Lehnert *et al* (1985) which showed that the numbers of particles washed from the pleural spaces of rats, after their instillation in the lung, were essentially the same as those harvested from a group of control rats. They concluded that smooth, spheroidal polystyrene particles with a geometric diameter of $= 2 \mu\text{m}$ do not pass to the pleural space, even when deposited in the lung in high numbers. However, contradictory results have been observed as discussed in Section 3.3.

3.2. Fate of *C.parvum* organisms following intratracheal instillation

During *C.parvum*-induced alveolar inflammation, many *C.parvum* organisms were observed inside alveolar macrophages and PMNs. However, neither pleural macrophages nor PMNs were found to contain *C.parvum* organisms. This generally supports the contention that intratracheally instilled particles do not reach the pleural space.

3.3. Transmission electron microscope (TEM) evidence of transfer of asbestos fibres from the airspace to the pleural space

A TEM study was carried out to observe whether any asbestos fibres could be seen in the pleural macrophages or PMNs after rats were exposed, by long term inhalation followed by 1 year recovery, by short term inhalation or by intratracheal instillation, to different asbestos types alone or in combination with TiO_2 or quartz. The pleural leukocytes were carefully examined by TEM for internalised asbestos fibres and although large numbers of cells were scanned, no fibres were ever found in the pleural macrophages or PMNs. Meanwhile, asbestos was easily discerned in alveolar macrophages.

Experimental findings on the translocation of particles or fibres from the lung to the pleural space are not all in agreement. Sebastien *et al* (1979)

demonstrated asbestos and other fibres at visceral pleural and parietal sites, and in pleural fluid. Miller *et al* (1989) found that after inhalation of glass fibres and ceramic fibres, cells containing partly phagocytosed fibres were present in the pleural space as early as 3 days post-exposure. The majority of fibres observed in the pleural lavage supernatants by scanning electron microscope appeared shorter than 8 μm . Holt (1983) reported the translocation of inhaled chrysotile to the pleura by "drift" towards the pleura within macrophages. Pleural migration of chrysotile fibres after intratracheal injection in rats was also demonstrated by Viallat *et al* (1986) and they found that the size and quantity of chrysotile fibres that reached the pleural cavity of test rats was time-dependent, with two peaks on day 7, mean number = 0.96×10^5 ; mean length = 1.3 μm , and on day 21 with mean number = 1.3×10^5 and mean diameter = 0.3 μm .

However, Oberdoerster *et al* (1983) could not detect amosite fibres in the pleura after rats were intratracheally instilled. Light microscope and TEM study presented by Dodson and Ford (1985) also showed no evidence of amosite fibres within the pleura or subpleural regions after instillation and macrophages on the pleural surface were found to contain no fibre.

The present study indicated that beads with a geometric diameter of 0.77 μm do not reach pleural space at one day or one month after they are deposited in the lung. This diameter is less than the length of chrysotile fibres observed in pleural spaces, i.e. up to 1.3 μm (Viallat *et al*, 1986). The contention that particles do not easily transfer is also supported by the *C.parvum* study. It is suggested that, in the animal model used in the present study, there is no detectable translocation of asbestos fibre into the pleural space from the lung after instillation.

The morphology of the pleura limits the translocation of particles from the lung to the pleural cavity. The visceral pleura is thick and is composed of five different layers (Staub *et al*, 1985). The present TEM study on pleural structures confirmed these histological patterns. This represents a formidable barrier to movement of particles from the alveolar to the pleural spaces which particles are unlikely to breach easily.

However, transportation of particles into the pleura may be facilitated by specific structures on the pleura, such as milky spots, crevices and pleural stoma (Kanazawa, 1985). But the present study failed to demonstrate the role of these structures in transfer of asbestos fibres into the pleura.

Intratracheal instillation and inhalation of asbestos gives very different pictures (Oberdoerster *et al*, 1983; Davis *et al*, 1986b and the present study) to

that seen with intrapleural injection of asbestos (Sahn and Antony, 1984; Edwards *et al*, 1984; Shore *et al*, 1983) in terms of pleural cell population changes, as well as pleural pathological changes. This could be taken as evidence that asbestos does not enter the pleural space in the case of the first two but that this response is a reactive one to adjacent inflammation.

Dodson and Ford's findings (1985) on amosite asbestos intratracheal instillation study indicated that pleural morphological changes following asbestos exposure included: (1) pleomorphic changes in mesothelial cells ranging from slightly cuboidal, to protruding "columnar-like" cells, to more bizarre forms; (2) early proliferation of the mesothelial cells; (3) distortions of the basal lamina. Limited evidence in the present study also showed the presence of mesothelial cell injury caused by asbestos exposure.

4. *In vitro* studies on pleural mesothelial cells

Mesothelial cells are highly susceptible to damage from agents that they do not normally encounter, including asbestos, silica and foreign protein. All these cause swelling of the cells, clubbing of microvilli, and separation of the cells from each other and from the basal lamina, resulting in exfoliation (Herbert, 1986). We do not believe that there is substantial transfer of fibres sufficient to cause this type of injury. However, we have reported that inflammatory alveolar leukocytes can damage epithelial cells in culture (Donaldson *et al*, 1987_b). We set out to determine whether pleural leukocytes could be activated to cause damage to mesothelial cells following I/T fibres. Studies were therefore carried out to investigate the interaction between pleural leukocytes and mesothelial cells.

4.1. Injurious effects on mesothelial cells *in vitro* caused by pleural leukocytes from rats exposed to asbestos by intratracheal instillation

In a similar pattern to the earlier part of the present study (Section 1.2.1) in this sequence of experiments there were increasing numbers of macrophages and eosinophils in the pleural spaces at different times post crocidolite I/T. However, differently to the first experiment was the absence of PMN in the pleural spaces following asbestos I/T.

We have previously used detachment injury of epithelial cells to demonstrate the potentially injurious properties of inflammatory leukocytes (Donaldson *et al*, 1987_b). Using the standard assay for measuring cell detachment, we were able to demonstrate mesothelial cell injury caused by pleural leukocytes from rats intratracheally exposed to asbestos. The results revealed that asbestos-derived pleural inflammatory cells caused considerable mesothelial cell detachment but not lysis injury, an effect we found previously to be

protease-mediated (Donaldson *et al*, 1987_b). The changes of cell components were not the explanation of the findings since there is no significant difference of pleural leukocyte components between controls and asbestos I/T animals. Further studies indicated that mesothelial cells were susceptible to both trypsin and elastase in terms of detachment injury.

Neutrophil-induced detachment injury of rat pulmonary alveolar epithelial cells has been reported by Ayars *et al* (1984) and Simon *et al* (1986) who showed that stimulated PMNs produced marked target cell detachment without lysis. However, PMNs are not involved in mesothelial cell detachment observed in the present study because there were no PMN in the pleural leukocyte populations. It must be presumed that pleural macrophages or other cell components, such as mast cells and eosinophils, may also contain proteases (Henson *et al*, 1988) ^{and are} able to cause mesothelial cell detachment. These may be stimulated and released in increased amounts while macrophages or other cells are triggered by inflammatory messages from adjacent asbestos-exposed parenchyma. Since this activity of pleural leukocytes declined rapidly, it is probably overshadowed by newly recruited macrophages or other pleural components which are less responsive to the alveolar-derived inflammatory signals or are able to produce more protease inhibitors.

The present study also provided evidence that elastase may play a role in the mesothelial cell detachment injury caused by inflammatory pleural leukocytes. This is consistent with other observations: Ayars *et al* (1984) demonstrated that elastase and collagenase exert effects on target cell detachment and PMN-mediated detachment was markedly inhibited by neutral protease inhibitors and elastase inhibitor. From the effects of active proteases, the mechanisms of detachment is through the action of granule neutral proteases, rather than toxic oxygen metabolites, and is probably due to degradation of the extracellular matrix of the target cells, as suggested by Ayars *et al* (1984) and Donaldson *et al* (1987_b). Simon *et al* (1986) also suggested that damage to pulmonary alveolar epithelial cells caused by stimulated PMNs was oxygen metabolite-independent. Brown and Donaldson (1988) found, in an investigation of the role of leukocytes in connective tissue derangements in the lung, that inflammatory cells which contained more than 75% of PMNs caused consistently and substantially more degradation of fibronectin than controls on a per cell basis and serine proteases are responsible for this matrix degradation.

Detachment could lead to desquamation and exfoliation of mesothelial cells *in vivo* and increased permeability of the pleural barrier as well as damage to the

lubrication function of the mesothelium. As reported, mesothelial cells were not present in washing from normal pleural spaces (Spriggs and Boddington, 1968), but may be abundant in many forms of effusion (Herbert, 1986). In our TEM study, evidence was presented that, during long term inhalation exposure of rats to asbestos, there was exfoliation of mesothelial cells from their matrix.

Pleural inflammation in humans would be much less intense than the inflammation produced here experimentally. Thus, the burden of inflammatory cells may be insufficient for such an extreme detachment response as demonstrated here *in vitro*, and the potential to cause injury may also be ameliorated by anti-protease defence mechanisms in the pleura (Donaldson *et al*, 1988_b). However, under these conditions low level attack on the mesothelial cells could still lead to the increased permeability of the mesothelial membrane and accumulation of fluid with high protein content as described in some inflammatory processes and tumours of the pleura (Rennard *et al*, 1985).

4.2. Injurious effects towards mesothelial cells by pleural leukocytes from rats with *C.parvum*-induced pleural inflammation

Intratracheal instillation of *C.parvum* resulted in both bronchoalveolar and pleural inflammation characterized by recruitment of PMNs into those spaces by day 1 and recruitment of macrophages by day 5. *C.parvum*-elicited alveolar leukocytes caused considerable detachment of mesothelial cells, which was consistent with the findings of Donaldson *et al* (1988_b) despite the fact that different target cells were used in the present model. Pleural leukocytes from rats 1 day following *C.parvum*- exposure which consist of 30% PMN, resulted in substantial mesothelial cell detachment in a cell concentration-related manner. However, the leukocytes from rats 5 days after *C.parvum* I/T which contained almost no PMN, had no effect in causing detachment injury of mesothelial cells. However, those cells caused modest target cell lysis.

PMNs exert injurious effects on rat alveolar epithelial cells (Simon *et al*, 1986; Donaldson *et al*, 1988_b), human A549 pneumocytes (Ayars *et al*, 1984) and human endothelial cells (Cronstain *et al*, 1986). The present study indicated that pleural PMNs have the potential to cause mesothelial cell detachment since detachment injury is related to severity of neutrophil pleuritis. However, the possibility that pleural macrophages or other components may play a role in this mesothelial cell injury cannot be excluded since there are very different pleuritis induced by *C.parvum* and asbestos in terms of cell populations. It would be anticipated that different mechanisms may lead to these similar phenomenon. Nevertheless, it is still not clear why pleural leukocytes caused mesothelial cell

lysis injury by day 5 when newly recruited macrophages accounted for the most of pleural population.

4.3. Pleural lavage from rats exposed to crocidolite asbestos stimulates mesothelial cell proliferation *in vitro*

Various types of inflammatory agent, such as asbestos, microorganisms and endotoxin have been shown to induce a rapid desquamation of the mesothelium (Bignon and Gee, 1985). Under certain circumstance, mesothelial cells would proliferate to repair themselves, leading to hyperplasia (Herbert, 1986). In addition, pleural leukocyte-derived growth factor could also be important in the hyperplasia. This may lead to pleural fibrosis and mesothelioma in some cases.

In the present study, pleural lavage fluids were tested for their stimulating effect on mesothelial cell proliferation. The pleural lavage fluid by day 30 after instillation stimulated the proliferation of mesothelial cells. The results agree with the observation of Fotev *et al* (1987) that peritoneal wound exudates stimulated DNA synthesis in normal quiescent mesothelial cells.

Inflammatory cells could stimulate a proliferative response in the mesothelium and lead to the mesothelial hyperplasia described in chronically irritated pleura (Whitaker *et al*, 1982_b), and also the development of mesothelial proliferative lesions and mesothelioma in mineral fibre-exposed individuals (Davis *et al*, 1986_a). This type of response is likely to be mediated by macrophages since the cells have been shown, extensively, to be able to stimulate growth in a wide variety of mesenchymal cell types (Martin *et al*, 1981; Glenn and Ross, 1981). Fotev *et al* (1987) found that the cells principally responsible for stimulation of DNA synthesis in mesothelial cells by peritoneal wound exudates were the macrophages. They confirmed that cultured macrophage-conditioned media induced an increase in mesothelial replication and postulated that exudate macrophages secrete mitogenic factor(s). Lemaire *et al* (1983) reported that asbestos exposure enhances the release of fibroblast growth factor by sheep alveolar macrophages. In the present study, the result may imply the release of mesothelial cell growth factor by pleural macrophages in response to asbestos exposure. Further work is clearly required to characterise this mediator.

Presumably, if pleural macrophages are present in increased numbers, or in an activated state, during asbestos-induced injury, then they could release these factors in sufficient quantities to produce proliferative lesions in the mesothelium. The present study supports evidence on this point: thirty days following I/T asbestos, there was substantial recruitment of macrophages into the pleural space. These may exert mesothelial cell mitogenesis via the production of growth factors.

This observation may shed light on chronic mesothelial hyperplasia and possibly, mesothelioma, in mineral fibre exposed individuals.

5. Bronchoalveolar leukocyte response to asbestos

As a major effector system in pulmonary pathobiology, bronchoalveolar leukocytes have attracted abundant attention as to changes following exposure to asbestos, eg. plasminogen activator production in asbestosis (Cantin *et al*, 1989), IL-1 and TNF production in response to asbestos exposure *in vitro* (Rosenthal *et al*, 1989; Dubois *et al*, 1989) and *in vivo* (Bissonnette and Rola-Pleszczynski, 1989; Driscoll *et al*, 1990,) and so on. The present study was directed mainly at pleural leukocytes but to compare the responses of pleural leukocytes and alveolar leukocytes to asbestos exposure, cytokine production by bronchoalveolar leukocytes following I/T asbestos was assessed.

5.1. Cytokine production by alveolar leukocytes and effect of I/T asbestos

5.1.1. Production of TNF and IL-1 by normal alveolar leukocytes

It has been reported that there was no TNF activity in supernatants of unstimulated rat alveolar macrophages and that stimulation with LPS was required to induce production and release of high levels of TNF (Becker *et al*, 1989; Adams and Czuprynski, 1990; Martinet *et al*, 1988). As with TNF, alveolar macrophages produce IL-1 only in response to a variety of stimulants including LPS (Oppenheim *et al*, 1986; Wewers *et al*, 1984).

Our rat alveolar macrophages produced TNF and IL-1 *in vitro* without addition of stimulants. Since a relatively low level contamination of endotoxin (up to 100 pg/ml) was found in the media used in assays, then these could contribute to the spontaneous release of both TNF and IL-1 (Dinarelli, 1984). This possibility has been ruled out for observed TNF production since our endotoxin-depleting study indicated that removal of contaminated endotoxin from the media does not affect TNF production by pleural leukocytes. Furthermore, we confirmed that the normal alveolar macrophages were able to produce significantly enhanced TNF and IL-1 activity in response to 100 ng/ml of LPS.

5.1.2. Alveolar leukocyte population after I/T asbestos

Following intratracheal crocidolite asbestos, there was an acute recruitment of PMN at 1 day. This is consistent with the work of Donaldson *et al* (1988_b) revealing that the predominant cell types in lavage fluid were macrophages in control rats and macrophages plus PMNs in rats exposed to chrysotile. However, they found that basophils and eosinophils never exceeded 1% of the total cells. This contrasts with the present findings that eosinophils exceeded 15% by day 3. This may be explained by the different kinds of asbestos or different strains of

animals. Xaubet *et al* (1986) and Robinson *et al* (1986) have described the increased levels of eosinophils in the bronchoalveolar lavage of asbestos-exposed individuals. Oberdoerster *et al* (1983) reported that after amosite I/T, PMNs in lung lavages of rats increased dramatically by day 1, and decreased from day 3 onwards. Meanwhile, the proportions of macrophages changed in a complementary way. Similar patterns of variation of PMNs and macrophages were found in the present study.

One month following I/T asbestos, no important changes in bronchoalveolar leukocyte populations were observed across asbestos doses except the presence of a small proportion of PMNs.

5.1.3. Production of TNF by alveolar leukocytes after I/T crocidolite asbestos

Studies on alveolar macrophages exposed to asbestos *in vitro* indicated that the addition of 30 $\mu\text{g/ml}$ crocidolite asbestos to alveolar macrophage pre-incubated with RPMI and serum resulted in a significant increase in TNF production when compared to control (Rosenthal *et al*, 1989). Another *in vitro* study also showed that chrysotile asbestos augmented TNF production by alveolar macrophages in a concentration-dependent fashion (Dubois *et al*, 1989).

The present study in keeping with these previous findings indicated that there is an increase in TNF production by alveolar leukocytes. An asbestos-related dose effect on TNF production by these cells was observed, although lower doses did not enhance TNF release sufficiently to attain statistical significance. The absence of an increase on day 1 may be explained by a substantial recruitment of PMN which may remove TNF via receptor (Shalaby *et al*, 1987). The increase in TNF production by the alveolar leukocytes is correlated with the instilled dose of asbestos. This could be important for fibrosis in the alveolar region of asbestos exposed individuals since TNF exerts potent growth factor activity for many types of human fibroblasts (Vilcek *et al*, 1986) and contributes to fibroblast growth activity stimulated by other mineral dust as confirmed by Dubois *et al* (1989). Palombella *et al* (1987) have shown that TNF can increase the number of epidermal growth factor (EGF) receptors on human FS-4 fibroblasts and that this increase may be related to the mitogenic action of TNF on these cells. In a further study, Palombella *et al* (1988) found that there was a synergistic interaction between TNF and EGF and platelet-derived growth factor in promoting DNA synthesis and cell division of fibroblasts. *In vivo*, local release of TNF causes a focal accumulation of fibroblasts and collagen (Piquet *et al*, 1990_b). It is also suggested that TNF can provoke acute pulmonary vascular endothelial injury through increasing permeability of endothelial cell monolayers (Goldblum *et*

al, 1989; Brett *et al*, 1989) as well as influencing human fibrinolytic system (Silverman *et al*, 1990).

As TNF infusion results in alveolar damage, the extensive secretion of TNF in the lung during exposure to asbestos could be an important step in the development of fibrosis. Abnormally increased secretion of TNF α by alveolar macrophages in coal worker's pneumoconiosis has been reported by Lassalle *et al* (1990). They concluded that alveolar macrophages are involved in chronic inflammatory lung reactions to mineral dusts, partly through TNF secretion. Piguet *et al* (1990_a) confirmed the requirement of TNF for development of silica-induced pulmonary fibrosis in a mouse model. They showed that a single instillation of silica lead to a marked increase in the level of lung TNF messenger RNA lasting for more than 70 days. Silica-induced collagen deposition was almost completely abolished by anti-TNF antibody, and was significantly increased by continuous infusion of mouse rTNF.

TNF was also found to be an inducer of the release of IL-1 by endothelial cells (Nawroth *et al*, 1986), by mononuclear cells (Dinarello *et al*, 1986) and by fibroblast (Elias and Reynolds, 1990). IL-1 has synergistic effects with TNF in many aspects (Wankowicz *et al*, 1988; Elias and Reynold, 1990) which would favour inflammation and fibrosis and so its level in the lung with asbestos deposition is of great potential importance for long-term pathology.

5.1.4. Alteration of IL-1 production by alveolar leukocytes after I/T crocidolite asbestos

The alveolar leukocyte components changed following asbestos instillation, the most important change was an increased percentage of PMNs and eosinophils from day 1 to day 14. Since macrophages are a major source of IL-1 (Ohkawara *et al*, 1989; Dinarello, 1984), the decreased IL-1 activity might result from a decrease in the percentage macrophages or from the production of IL-1 inhibitor by PMN (Tiku *et al*, 1986_b).

However, the decreased IL-1 activity at day 14 is difficult to explain fully by the change in cell components since the percentage of macrophages had recovered to control level. This continuous decrease may be due to injury of macrophages by asbestos directly or due to the influence of an increased number of eosinophils.

The considerable enhancement of IL-1 activity released by alveolar leukocytes one month post instillation could result partly from the recruitment of macrophages. As revealed by Elias *et al* (1985), denser alveolar macrophages which represent less mature cells elaborated more IL-1 than less dense alveolar

macrophages. Rich *et al* (1989) and Elias *et al* (1985) have also shown that human alveolar macrophages produce less IL-1 in response to LPS than do monocytes. Kagan *et al* (1989) demonstrated that significantly greater proportions of alveolar macrophages were recoverable in the highest density fractions on day 7 and day 17 after crocidolite or chrysotile inhalation exposure, compared to sham exposure. In the present study, possibly, newly recruited macrophages may accumulate at sites of asbestos deposition, as supported by the observation of Warheit *et al* (1985) that asbestos-generated chemoattractants for alveolar macrophages were present in the lung lining fluid during inhalation exposure. Therefore, the IL-1 activity produced by these accumulated cells would be enhanced.

A large body of literature has accumulated in recent years implicating IL-1 in the pathogenesis of mineral dusts and asbestos-induced lung abnormalities. Studies indicated an increasing release of IL-1 by alveolar leukocytes *in vivo* exposed to particles, such as coal dusts (Kusaka *et al*, 1990_a) and quartz (Dirscoll *et al*, 1990₁). Lassalle *et al* (1990) observed higher levels of IL-1 secretion by alveolar macrophages from patients with coal worker's pneumoconiosis than in those from healthy controls.

As reported by Rosenthal *et al* (1989), chrysotile and crocidolite asbestos markedly stimulated IL-1 production by alveolar macrophages with stimulation with LPS prior to fibre exposure. Enhanced IL-1 activity following asbestos inhalation in rat was reported by Hartmann *et al* (1984) and Kagan *et al* (1985_a). The present study showed that one month after I/T asbestos, IL-1 production increased dramatically and a dose-related effect of asbestos was observed.

Although PMNs have been found to synthesize and release IL-1 in culture, this production required stimulation with opsonized zymosan particles (Canning and Neill, 1989) or PMA (Tiku *et al*, 1986a). Murine eosinophils are also able to express IL-1 mRNA when stimulated with LPS (Pozo *et al*, 1990). However, alveolar PMN and eosinophils seem not to be responsible for the altered IL-1 activity seen during asbestos exposure.

Human rIL-1 was found to stimulate the synthesis and accumulation of type I procollagen chains, type I procollagen messenger RNAs and growth of fibroblasts *in vitro* (Postlethwaite *et al*, 1988). Hartmann *et al* (1984) showed that asbestos-associated augmented release of IL-1 stimulates fibroblast DNA synthesis and lymphoproliferation. Since IL-1 may modulate the functions of fibroblasts as well as other inflammatory cells, the persistent augmented release of IL-1 at sites of inflammation may contribute to fibrogenesis and fibrosis after asbestos

deposition in the lungs.

IL-2 is also able to induce mouse thymocyte proliferation (Kagan *et al*, 1985_a) and sometimes it is co-produced with IL-1 by macrophages (Hartmann *et al*, 1984). To exclude the possibility in the present study that IL-1 activity measured by using mouse thymocyte proliferation assay was caused by the presence of IL-2, IL-2 activity was measured and none was detected.

5.1.5. Effect of mineral dusts and asbestos *in vivo*, individually or in combination, on TNF and IL-1 production by alveolar leukocytes

5.1.5.1. Alveolar leukocyte components after I/T mineral dusts and asbestos

The present study indicated that one month after instillation of compact mineral dust and asbestos, individually or in combination, the most dramatic change was a substantial recruitment of alveolar leukocytes. The total number of the leukocytes was more than six times greater in the quartz treated- group and ten-fold more in the quartz plus crocidolite group. Another main change induced by dust instillation was the increased percentage of PMN, being 41% in the quartz treated group and 27% in quartz plus crocidolite group. The observation implied that quartz is a very strong stimulant in the lung, which is consistent with the findings of Donaldson *et al* (1988_c) on inflammatory cell recruitment to quartz-instilled lung. In another study, Donaldson *et al* (1990) showed that, during exposure to airborne quartz, there were substantial increases in alveolar macrophages and PMNs. Driscoll *et al* (1990₁) also revealed that, 28 days after intratracheal instillation, dramatic increases in both alveolar macrophages and PMNs were observed in the quartz group; TiO₂, by contrast, caused only a moderate increase of both kinds of cells.

It is likely that, within working situations, there is mixed exposure to asbestos and other mineral dusts. The present study showed that the combination of quartz and crocidolite in the lungs resulted in dramatically increased cellular response compared to control and groups receiving single dust. This gives further support to the findings of Davis *et al* (1991) that quartz plus asbestos greatly increased fibrosis and pulmonary tumours above that produced by asbestos alone during inhalation exposure.

5.1.5.2. Effect of intratracheal mineral dusts and asbestos on TNF and IL-1 production by alveolar leukocytes

One month post mineral dusts and asbestos I/T, individually or in combination, the most significant increase in TNF production by alveolar leukocytes was stimulated by TiO₂ alone and TiO₂ plus crocidolite; quartz decreased TNF production. However, IL-1 production by alveolar leukocytes was

increased by any dust treatment, individually or in combination, TiO_2 stimulated the biggest enhancement of IL-1 elaboration, quartz stimulated the least.

The present study indicates that TiO_2 -elicited alveolar macrophages produce much higher TNF and IL-1 activities in culture than quartz or crocidolite-elicited leukocytes. The results are consistent with the finding of Kusaka *et al* (1990_a) which indicate that TiO_2 is capable of stimulating alveolar macrophage IL-1 production. The increased cytokine production by alveolar leukocytes after I/T 2.5 mg TiO_2 may represent the effect of the overload of particulates as Driscoll *et al* (1990₂) described occurring with 2-10 mg TiO_2 in 200g rats. Crocidolite stimulated IL-1 production by alveolar leukocytes and this agrees with the study of Hartmann *et al* (1984) showing crocidolite inhalation is followed by enhanced IL-1 release by alveolar macrophages. Our finding that intratracheal quartz resulted in decreased TNF is not consistent with the findings of Driscoll *et al* (1990₁) but the dose used here (2.5mg) was below effective dose (10mg) found in that study. The fact that a mixed population, with up to 40% PMN, was used in the present study whereas Driscoll *et al* determined the response of separated macrophages could also contribute to the different findings. PMN may not only exert a diluting effect on macrophage number which are the most potent cell source of both TNF and IL-1 (Driscoll *et al*, 1990₁), but can also release prostaglandin E_2 which has been reported to down-regulate TNF production (Kunkel *et al*, 1988). In addition, PMN may also internalize TNF via their TNF receptors (Shalaby *et al*, 1987).

Although synergistic effects were observed in the combination of quartz and crocidolite on cell recruitment, no dramatic changes in cytokine production on a per cell basis could be detected.

5.1.6. *In vitro* TNF and IL-1 production by alveolar macrophages in response to mineral dust and asbestos, individually or in combination

Very similar patterns of TNF and IL-1 production by alveolar macrophages were found. The co-culture of quartz or crocidolite, but not TiO_2 , with alveolar macrophages caused increased release of both IL-1 and TNF. The combination of crocidolite with TiO_2 had an additive effect with respect to cytokine release by the macrophages, but dual exposure to crocidolite plus quartz had no such effect. These results were consistent with other workers' observations that quartz normally acts as a stimulant of TNF and IL-1 production while TiO_2 has limited or little effect (Driscoll *et al*, 1990₁; Dubois *et al*, 1989; Kusaka *et al*, 1990). In the present study, the combination of quartz and crocidolite lead to reduced cytokine production by the macrophages compared to quartz alone, possibly as a

result of a toxic effect.

These results revealed very different response of alveolar macrophages to mineral dusts and asbestos *in vitro* and alveolar leukocytes *in vivo*. Clearly, *in vivo* exposure to mineral dusts or asbestos causes a mixed cellular response with recruitment, activation, turnover, interaction with each other and death, to varying degree, which cannot be reflected in an *in vitro* study.

Since alveolar macrophages are capable of releasing cytokines to modulate the activities of inflammatory and immunocompetent cells, they have the potential to play a key role in the initiation and perpetuation of inflammatory responses within the lung. TNF and IL-1 have been documented to have pro-inflammatory activities (Sections 5.1.3 and 5.1.4). The local release of these cytokines can influence the initiation and progression of the inflammatory response and favour the development of pulmonary fibrosis (Piguet *et al*, 1990_a; Borm *et al*, 1988; Lassalle *et al*, 1990).

With regard to the present findings, it is clear that cytokine production by both alveolar and pleural leukocytes differs in response to instilled mineral dusts or asbestos. *In vivo* exposure to crocidolite caused IL-1 production to be decreased at earlier stages by both alveolar and pleural leukocytes whilst one month after exposure, the IL-1 release was enhanced. However, the study on TNF production after exposure to dusts alone or in combination on alveolar and pleural leukocytes gave a very different pattern: alveolar leukocytes released increased TNF activity after exposure, whereas pleural leukocytes released decreased TNF activity compared to control. There was a considerable synergistic interaction between mineral dusts and asbestos in inducing pleural leukocytes to release TNF, but there was no such effect in stimulating alveolar leukocytes.

These findings demonstrate that the pleural and alveolar spaces are different in response to deposition of asbestos or other mineral dusts. The pleural cavity has a limited opportunity to encounter inhaled or intratracheally instilled mineral dusts or asbestos fibres (Oberdoerster *et al*, 1983). This may mean that pleural leukocytes are affected by dusts or fibres by indirect ways. Alveolar leukocytes, by contrast, interact with dusts or fibres directly.

In addition, alveolar leukocyte components and pleural leukocyte components are very different since pleural leukocytes contain many mast cells and eosinophils which are not resident in the normal alveolar space. From the point of pathological changes, pleural plaques and mesothelioma are distinct from alveolar pathological changes resulting from asbestos exposure (Craighead *et al*, 1982; Wagner, 1986). The present study suggests that there are, in the short

term, very different inflammatory responses in the two spaces with regard to cell recruitment and mediator release. This difference in inflammation could be important in understanding the different pathological responses found in the two regions.

5.2. TNF and IL-1 production by alveolar macrophages in response to long and short fibre amosite exposure *in vitro*

The present study indicates that long fibre amosite stimulated alveolar macrophages to release substantially increased TNF and IL-1 activity in culture, whereas short fibre amosite had no significant stimulating effect.

Previous studies have shown differences in the pathogenicity of the long and short fibre amosite samples (Davis, 1986_a) with long amosite being more active in inducing lung fibrosis, tumours and mesotheliomas than the short amosite. Donaldson *et al* (1989_a) found that the ability of amosite samples to cause inflammation, as judged by macrophage and PMN recruitment into the mouse peritoneal cavity, was ranked in the order: long fibre > UICC sample (intermediated in fibre size) > short fibre. Adamson and Bowden (1990) reported that instilled long crocidolite (>2.5 μm) induced pulmonary fibrosis whereas the same dose of short fibres did not and suggested that pulmonary fibrosis after long fibre administration appears unrelated to alveolar macrophage secretion of growth factor and is probably caused by fibre penetration into the peribronchiolar tissue, where interstitial macrophage activation may occur. The present study provides additional evidence for the enhanced pathological activity of long fibre amosite. The study shows increased cytokine production by alveolar macrophages exposed to asbestos, which is consistent with other workers' findings (Dubois *et al*, 1989; Rosenthal *et al*, 1989) and reveals that asbestos fibre length appears critical in this event. These results suggests that the increased pathology caused by long fibres may be related to the ability of long fibre to cause cytokine secretion.

5.3. The effect of opsonization of dusts on TNF release by alveolar macrophages

Immunoglobulin G (IgG), as a component of the lung lining fluids, has been demonstrated to change secretion by alveolar macrophages (Ward *et al*, 1983; Brieland *et al*, 1987) and enhance asbestos-stimulated superoxide anion production by the alveolar macrophages (Scheule and Holian, 1989). In view of these findings, a study on the effect of opsonized asbestos and other dusts, on TNF secretion was carried out. The results showed that, compared to the control ("PBS-opsonized" group), the IgG-opsonized group had a substantial greater ability to stimulate TNF release by alveolar macrophages. Heat-inactivated rat serum

had a slight enhancing effect on the two most active dusts, long fibre amosite and quartz. This may reflect the effect of a non-specific protein coating. However, BSA had no such effect compared to control.

Fibronectin-opsionized long and short fibre amosite had moderate effect in stimulating TNF release. Amongst the functions of fibronectin are cell-to-cell attachment, cell adherence to basement membranes and as a matrix for cell migration and extracellular matrix reorganization (Rossman *et al*, 1990). Since leukocytes may process receptors for fibronectin, some stimulation by fibronectin-coated fibres would be anticipated.

Nevertheless, the most significant effect was caused by IgG opsonization. IgG opsonization caused all of the dusts, including TiO_2 , to exert an increased effect in stimulating TNF production by alveolar macrophages. A further study using different doses of IgG to opsonize demonstrated that long fibre amosite was most susceptible to opsonization by IgG while TiO_2 had the least response to the opsonization.

IgG has been shown to considerably enhance the production of superoxide anion production by guinea pig alveolar macrophages (Scheule and Holian, 1989) and reactive oxygen metabolites by human macrophages (Nyberg and Klockars, 1990) in response to chrysotile asbestos. The workers suggested that this is an Fc-receptor-mediated and relatively specific phenomenon. Pulmonary alveolar macrophages have been shown to have receptors on their surface for IgG (Daughaday and Douglas, 1976; Tizard, 1988). Therefore, particles opsonized with antibody can bind to macrophages and stimulate their metabolism if sufficient Fc domains interact with Fc receptors to effectively crosslink them (Sibille and Reynolds, 1990).

An IgG dose-dependent enhancement of TNF production by alveolar macrophages was observed in the present study. Using pre-incubation and washing, the increased TNF production in the presence of IgG can be accounted for entirely by the IgG absorbed onto the asbestos surface. As suggested by Nyberg and Klockars (1990), immunoglobulins very likely interact with mineral dusts in a "nonimmunological", antigen-independent way and that the so-formed dust-immunoglobulin complexes amplifies the production of active metabolites by the inflammatory cell. However, increased phagocytosis caused by protein-coating of dusts in this phenomenon may also play part role.

The present study also showed that different dusts have different abilities to be opsonized with IgG, which indicates that characteristics of the dust surface are important in the ability to be opsonized by IgG. It is proposed that as more

toxic and pathogenic dusts, long fibre amosite and quartz have more surface charge or active areas (Valerio *et al*, 1987; Wiessner *et al*, 1990) which causes them to have a higher affinity for IgG molecules and so bind to.

Since asbestos induces increased expression of macrophage surface IgG-Fc receptors (Tetley *et al*, 1976), the dramatic enhancement of long fibre amosite-induced TNF production by alveolar macrophages observed in the present study resulted, maybe partly, from increased IgG Fc receptors on the macrophage surface which would amplify the opsonized effect of IgG.

It is likely that the initial interaction between fibres and cells may be mediated by relatively nonspecific mechanism since, even unopsonized, long fibres are more stimulatory to TNF release than short fibres. The enhancement of TNF production may then result from opsonization of long fibres as a result of accumulation of IgG during inflammation. This could feed forward leading to enhancement of inflammatory response. Increased release and accumulation of other inflammatory mediators could also occur.

Abnormal levels of IgG in lavage of smokers have been documented (Bell *et al*, 1981) and this could be important in synergism between asbestos and smoking in causing lung disease. Patients with asbestosis also have a higher concentrations of IgG in their lavage than control (Bignon *et al*, 1978). Begin *et al* (1985_b) reported that in asbestos-induced alveolitis, there was an increase of all classes of immunoglobulin in the bronchoalveolar lavage fluid. The likely enhancement of inflammation that could result from the IgG-opsonization/ TNF release cycle could be of great importance for fibre-related lung disease and requires further investigation.

The complement system is also involved in many inflammatory situation (Bell *et al*, 1981; Tetley *et al*, 1976), and so the possible role of this system in IgG-opsonized fibre induced TNF release was also examined. The results showed that fresh rat serum treatment had no effect on IgG-opsonized amosite mediated enhancement of TNF release by alveolar macrophages. This suggests that IgG absorbed onto fibres is not sufficient in activating complement presumably because of failure to undergo the configurational changes in the Fc region that allow C_{1q} binding (Roitt *et al*, 1989).

6. Overview

In the present study, the changes in cytokines and other inflammatory mediators after exposure to asbestos or other agents were discussed separately. However, in general, these inflammatory mediators coexist and interact with each other during inflammation. An attempt to discuss these effects in combination

will therefore be made.

TNF increases the production of PAI-1 (Laschinger *et al*, 1990) and PAI-2 (Scarpati and Sadler, 1989) by endothelial cells. Silverman *et al* (1990) and Nieuwenhuizen (1990) demonstrated that the administration of rTNF α *in vivo* to cancer patients causes increased tPA activity first, then increased PAI activity by endothelial cells. Similar results were reported by Medcalf *et al* (1988) showed that human rTNF stimulated gene transcription of PAI-1 and -2 and simultaneously suppressed constitutive gene expression of tPA in a human fibrosarcoma cell. It was also demonstrated that TNF regulates mRNA for uPA and PAI-1 in human neoplastic cell lines (Georg *et al*, 1989).

IL-1 enhances mRNA levels for plasminogen activators, and their inhibitors, in human fibroblasts (Michel *et al*, 1989). Nachman *et al* (1986) reported the induction of PAI synthesis by IL-1 stimulation of human cultured endothelial cells. Medina *et al* (1989) found that IL-1 or TNF enhances the levels of PAI mRNA in bovine aortic endothelial cells.

TNF and IL-1 have no homology and there is no overlap in their receptor binding, but their activities show a remarkably high degree of overlap (Larrick and Kunkel, 1988). Also, IL-1 α and β and TNF α and β often are simultaneously produced in response to the same stimuli (Oppenheim *et al*, 1989). In addition, there is interaction between these two cytokines with respect of induction of synthesis: TNF was found to interact with endothelial cell receptors to induce release of IL-1 (Nawroth *et al*, 1986). Le *et al* (1987) have shown that TNF can induce the synthesis of both IL-1 α and β in human fibroblasts. Dinarello *et al* (1986) demonstrated rTNF is an endogenous inducer of IL-1.

Elias and Reynolds (1990) found that individually, rIL-1 and rTNF induced lung fibroblast expression of IL-1 activity, with rIL-1 being significantly more potent. Importantly, combining rIL-1 and rTNF synergistically stimulate the fibroblast IL-1 α production.

In view of the interaction and induction of these inflammatory mediators and cytokines by each other, it could not be ruled out that alterations in some mediators during asbestos exposure are modulated as a second order response to asbestos via other mediators. Nevertheless, in the present study, the fluctuation in release generally differs between mediators response to asbestos or other treatment. In addition, some efforts were made to exclude the possibility of cross-reactivities of inflammatory mediators assays.

7. Conclusion

7.1. The major outcome of the present study

The present study was undertaken to investigate the effects of asbestos deposition in the lungs, on pleural leukocytes, and the mechanism of any effects.

Since the rodent is a model used in many laboratories for extrapolation to humans, the rat model was chosen. As a fast, inexpensive and effective means to observe the acute effect of asbestos *in vivo*, intratracheal instillation of asbestos or other dusts was taken as the major approach in the present study although its shortcomings are obvious (Driscoll *et al*, 1990₁).

General

Intratracheal instillation (I/T) of asbestos resulted in the activation of pleural leukocytes characterized by modulation of fibrinolytic status, alteration of cytokine production and potential to cause injury to mesothelial cell *in vivo*. All of these occurred in the apparent absence (or very low level) of transfer of asbestos into the pleural space.

Specific findings

1. Normal pleural leukocytes secrete urokinase-type plasminogen activator inhibitor (PAI) *in vitro* while plasminogen activator (PA) activity is observed intracellularly.
2. Normal pleural leukocytes produce tumour necrosis factor (TNF) and interleukin-1 (IL-1) *in vitro* and the production is elevated by LPS stimulation.
3. Asbestos I/T provokes dramatic recruitment of alveolar inflammatory cells, such as neutrophils and macrophages. In the pleural space, moderate macrophage recruitment is found after a time-lag.
4. Asbestos I/T causes increased production of pleural leukocyte PAI, measured *in vitro* in an asbestos dose-dependent fashion, whereas intracellular PA activity is not altered.
5. Pleural leukocytes from rat intratracheally exposed to asbestos produce decreased TNF activity *in vitro*. However, IL-1 production by pleural leukocytes is decreased at earlier stages, then increased. There is an asbestos dose-related decrease in cytokine activity.
6. Intratracheal asbestos along with other mineral dusts considerably increases TNF release by pleural leukocytes in culture.
7. Pleural leukocytes from rats intratracheally exposed to asbestos cause mesothelial cell detachment injury *in vitro*.
8. Normal pleural lavage fluid inhibits TNF and IL-1 activity. Pleural lavage from rat long term after asbestos I/T stimulates proliferation of mesothelial cells.
9. A number of approaches were employed to try and detect asbestos fibres in the pleural region after intratracheal instillation. There was no evidence to

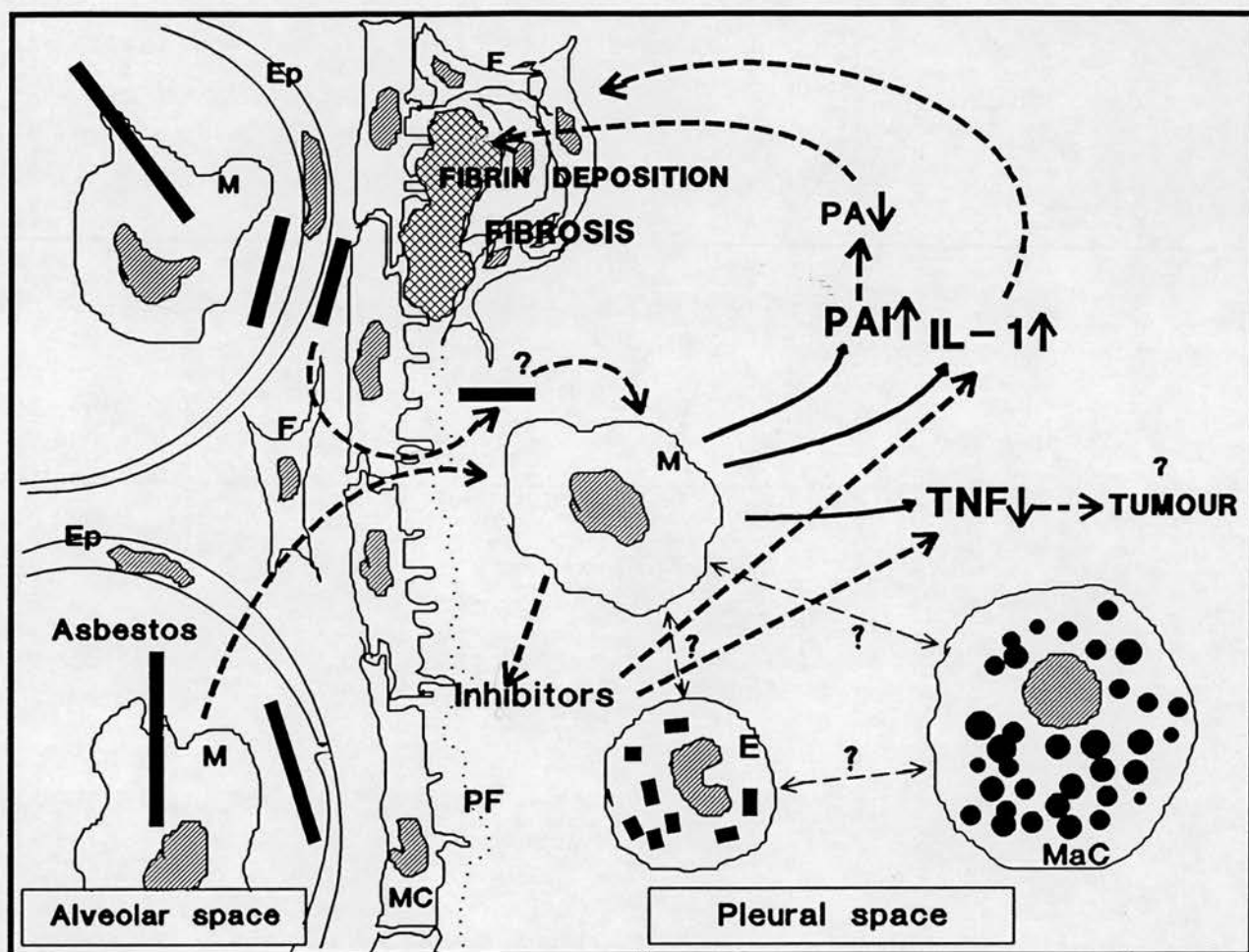


Figure D1 Diagram of the pleural response to intratracheal instillation of asbestos

M:- Macrophages; Ep:- alveolar epithelial cells; F:- fibroblasts; MC:- pleural mesothelial cells; PF:- pleural fluid; E:- eosinophils and MaC:- mast cells. Solid lines represent the results confirmed in the present study; dashed lines represent hypothetical relationship.

indicate

translocation of asbestos fibres to the pleural space, even during severe inflammation both in the lung and the pleura and at long times post instillation.

10. Normal alveolar leukocytes produce TNF and IL-1 activity *in vitro* and LPS enhances the production. In contrast to pleural leukocytes, asbestos I/T leads to increased TNF production. With IL-1 there is first a decrease, then an increase in IL-1 release by alveolar leukocytes.

11. Long fibre amosite asbestos and quartz exert greater activity in stimulating alveolar macrophages to release TNF than short fibre amosite and TiO_2 , respectively.

12. Opsonization of asbestos and mineral dusts by immunoglobulin G markedly increase their activity in stimulating TNF production by alveolar macrophages *in vitro*.

13. *C.parvum* I/T induces inflammation in both bronchoalveolar and pleural regions characterized by recruitment of neutrophils and macrophages. During the pleural inflammation, pleural leukocytes release decreased TNF and IL-1 activity, a transient increase of PAI activity and cause mesothelial cell detachment injury *in vitro*.

In Figure D.1 an attempt has been made to summarize, in diagrammatical form, some key results from the present study. Asbestos-stimulated alveolar macrophages may release diffusible mediator(s) which affect pleura macrophages, in terms of monokine production. In addition, asbestos fibres might penetrate into the pleural space and exert effects on pleural macrophages although we saw no evidence of this. In response to stimulation from the alveolar space, pleural leukocytes produce increased PAI and IL-1 as found in the present study. This may result, finally, in deposition of fibrin, as well as proliferation of fibroblasts so favouring fibrosis. As demonstrated in the present study, following deposition of asbestos in the airspace, pleural macrophages release decreased TNF. This phenomenon may result from an alteration in the level of inhibitor(s) in the pleural fluid. A sustained decrease in TNF levels could increase the possibility of tumour development. The interaction between different cell components in the pleural space following intratracheal asbestos are not clear.

7.2. Suggestions for future research

Some suggestions for further work have arisen from the present study with regard to the role of pleural leukocytes in asbestos-related diseases.

1. As a convenient means, intratracheal instillation is used throughout the study. However, since this is non-physiological model of exposure, a better reflection of

the events occurring after asbestos exposure would be gained from inhalation studies and these should be used to confirm the present observations. In addition, it would be very interesting to extend the time period to determine whether the pleural inflammation caused by asbestos in the airspace resolves or decays on cessation of exposure.

2. In view of present observations, the pleural space is an inhibitory environment in many respects. Further study would be required to investigate and characterize the inhibitors present in the pleural space and to examine their alteration during asbestos exposure in the hope of elucidating asbestos-related pleural pathological changes.

3. The present findings that there is no translocation of asbestos fibre from the bronchoalveolar space to the pleural space should be further confirmed. The use of radiolabelled asbestos fibre would be very useful in this context. In addition, which messengers play a role in passing the inflammatory signal to the pleural space needs to be clarified.

4. Although the present studies have shown that inflammatory pleural leukocytes cause injury to pleural mesothelial cells, the relationship between the leukocytes and mesothelial cells warrant further research.

5. The cellular basis of the synergism between asbestos and other mineral dusts in causing pleural injury following deposition in the bronchoalveolar region needs to be further confirmed and studied.

6. The modulation and interaction among different inflammatory mediators is not clear at the present. Further work should investigate the relationship between these mediators in the pleural space.

7. Immunoglobulin G (IgG) has been found to dramatically enhance the stimulating ability of dust on cytokine production by alveolar macrophages. The mechanism of such enhancement of dust activity by IgG requires further assessment.

8. In addition to the foregoing it is necessary to examine the full range of the functions which pleural macrophages, mast cells and eosinophils perform during asbestos-induced injury to the pleura. Oxidative metabolism, elaboration of other members of the interleukin family, growth factors, integrins and other key functional activities, need to be assessed to fully understand the role of the pleural leukocytes in pleural pathology caused by asbestos and other fibres.

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